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Lactic Acid Bacteria as protective and functional cultures for the enhancement of chestnut (*Castanea sativa* Mill.) processing chain

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ENGLISH ABSTRACT
Chestnuts are very perishable fruits, whose quality may be compromised during postharvest handling.
Damage can be caused both by insects and fungi. Water curing, a commonly used postharvest method, is based on soaking fruits in water typically for about one week. Factors that affect effectiveness of water curing have only been explained partially. A decrease in pH, likely imputable to a light fermentation caused by lactic acid bacteria, may inhibit the growth of moulds. In this study a *Lactobacillus pentosus* strain was selected for its ability to inhibit fungi, and used as a starter culture during water curing. As second goal, a reduction of the environmental impact of the process was evaluated by using water that had been re-cycled from a previous curing treatment. Experiments were performed on pilot as well as on farm scale. In all trials, microbial dynamics were evaluated by means of a polyphasic approach including conventional and molecular-based analyses. According to results, the employment of an adjunct culture appears as a very promising opportunity. Even if no reduction in the duration of the process was achieved, waters exhibited a minor microbial complexity and fruits did not lose the natural lustre after the process.
The second part of the project focused on the development of novel foods chestnut-based in order to seasonally adjust the offer and use the surplus of undersized production, providing, at the same time, a response to the growing demand for healthy and environmentally friendly products.
Broken dried chestnuts were employed to prepare purees to be fermented with six different strains of *Lactobacillusrhamnosus* and *Lactobacillus casei*. The fermented purees were characterized by a technological and sensorial point of view, while the employed strains were tested for their probiotic potential. Conventional in vitro tests have indicated the six lactobacilli strains as promising probiotic candidates; moreover, being the strains able to grow and to survive in chestnut puree at a population level higher than 8 Log CFU mL^{-1} along 40 days of storage at 4°C, the bases for the production of a new food, lactose-free and with reduced fat content, have been laid.
Subsequently, the effect of indigestible chestnut fiber and of chestnut extract on the viability of selected lactic acid bacteria strains was evaluated. Twelve strains were selected, on the basis of tolerance to low pH values and bile salts, and submitted to exposition to simulated gastric or bile juice in presence of chestnut extract with or without immobilization in chestnut fiber. The presence of chestnut extract proved to play a significant role on the gastric tolerance improvement of lactobacilli. The recorded protective effect could not be simply related to the starch or reducing sugars content. RP-HPLC demonstrated that in the chestnut flour, there are one or more hydrophobic peptides or oligopeptides, which specifically offer a marked resistance to simulated gastric juice, albeit present at low concentration. These beneficial effects proved to be dependent by the cultivar used to produce the flour. Moreover, were evaluated the suitability of chestnut extract as carrier for spray drying of two probiotic *Lactobacillus rhamnosus* strains and to develop a probiotic food chestnut based. The optimal settings for the spray-drying processes were defined and the loads of undamaged cells in the dried powders were quantified. Spray-dried cultures were incorporated into an anhydrous basis for chestnut mousse developed ad hoc. In this form, viable cells remained stable over 10^8 CFU g^{-1} during a 3 months long storage at 15°C. Sensorial analysis did not highlighted significant differences ($p< 0.05$) in preference between probiotic-supplemented and control mousses. Results suggest that chestnut mousse, a food product naturally rich in antioxidant compounds, may represent an excellent carrier for probiotics delivering.
Finally, chestnut flour was even evaluated for the production of bakery goods gluten-free. Microbial dynamics in the chestnut-based sourdoughs were evaluated by PCR-DGGE. The impact of fermentation on volatile organic compounds formation during sourdoughs maturation was evaluated by means of gas chromatography coupled to mass spectrometry (GC/MS).Members of the *Lactobacillus plantarum* group and *Pediococcus pentosaceous* dominated the sourdough ecosystems. Nevertheless, RAPD-PCR allowed recording a relevant genotypic biodiversity among strains coming from gluten-free flour combinations. A total of 59 volatile compounds were identified, mainly alcohols, esters, acids, aldehydes and ketones. Principal component analysis of samples at the beginning and at the end of ripening offered a good separation of the samples and highlighted the effect of fermentation on the sensorial profile.

ITALIAN ABSTRACT
Le castagne sono frutti piuttosto deperibili, la cui conservazione è costantemente minata dallo sviluppo di larve e funghi. Il trattamento più diffuso per la conservazione delle castagne è il cosiddetto processo di curatura o idroterapia, che consiste nell'immersione dei frutti in silos pieni di acqua a temperatura ambiente per circa una settimana. I fattori che influenzano l'efficacia del processo di curatura non sono ancora chiari. L'immersione in acqua provoca un abbassamento del pH riconducibile allo sviluppo di acido lattico, per processi presumibilmente fermentativi, che inibisce lo sviluppo di muffe.
In questo studio, un ceppo di Lactobacillus pentosus, caratterizzato da spiccata attività antifungina, è stato utilizzato come coltura adjunt durante il processo di curatura. Contestualmente è stata valutata la possibilità di ridurre l'impatto ambientale del processo di curatura attraverso l'utilizzo di acque recuperate da precedenti lavorazioni. Gli esperimenti sono stati condotti sia su scala pilota, sia su scala aziendale. In ogni set di esperimenti, il monitoraggio microbiologico convenzionale è stato affiancato da un approccio molecolare PCR-DGGE-based. In definitiva, l'impiego di una coltura adjunct batterica è apparso come una promettente opportunità. Anche se non si è registrata una diminuzione nei tempi di processo, si deve sottolineare che le acque trattate con coltura adjunct presentano una minore complessità microbica e i frutti non hanno perso la lucentezza dell'epicarpo dopo il processo.
La seconda parte del progetto ha puntato alla realizzazione di alimenti innovativi a base di castagna in grado di destagionalizzare l'offerta produttiva e valorizzare le eccedenze della filiera castanicola di scarso valore merceologico.

Castagne secche spezzate sono state impiegate per preparare puree fermentate con sei differenti ceppi di *Lactobacillusrhamnosus* e *Lactobacillus casei*. Le puree fermentate sono state caratterizzate da un punto di vista tecnologico e sensoriale, mentre i ceppi di batteri lattici utilizzati sono stati testati per il loro potenziale probiotico. Test *in vitro* hanno evidenziato il potenzialo probiotico dei sei ceppi di batteri lattici considerati; inoltre, tutte le colture si sono dimostrate in grado di crescere e sopravvivere in purea di castagna con livelli di popolazione superiori a 8 Log CFU mL^{-1} durante 40 giorni di conservazione a 4°C, gettando le premesse per la produzione di nuovi alimenti a ridotto contenuto in lattosio e grassi.

Successivamente, è stato valutato l'effetto della fibra e dell'estratto acquoso di castagna sulla vitalità di ceppi di batteri lattici selezionati. Dodici ceppi, selezionati giacché in grado di tollerare bassi valori di pH e la presenza di sali biliari, sono stati esposti al transito gastrointestinale simulato in presenza/assenza di estratto di castagna e con/senza immobilizzazione in fibra di castagna. L'estratto di castagna ha dimostrato di svolgere un ruolo significativo nel favorire la sopravvivenza dei lattobacilli all'ambiente gastrico. L'effetto protettivo registrato non è risultato correlato né al contenuto di amido, né a quello in zuccheri riducenti. L'analisi RP-HPLC ha evidenziato la presenza, nella farina di castagna, di uno o più peptidi anfipatici, in grado, seppure presenti a bassa concentrazione, di offrire protezione durante il transito in ambiente gastrico simulato. Inoltre, tale effetto protettivo si è dimostrato funzione della cultivar di castagna adoperata per produrre la farina.

In seguito, è stato valutato l'effetto protettivo dell'estratto di castagna durante l'essiccazione a mezzo spray-drying di due ceppi probiotici di *Lactobacillus rhamnosus* al fine di produrre cellule vive e vitali ma stabili per deidratazione da impiegare per lo sviluppo di un alimento probiotico a base di castagna.

Una volta definite le impostazioni ottimali per lo spray-drying, le colture essiccate sono state incorporate in una base anidra per la realizzazione di una mousse di castagna. In questa forma, le cellule sono rimaste vitali e stabili a 10^8 CFU g^{-1} durante tre mesi di conservazione a 15°C. L'analisi sensoriale non ha evidenziato differenze significative ($p<0.05$) tra le mousse probiotiche prodotte e il controllo. In altre parole, la mousse di castagna, un prodotto naturalmente ricco di composti antiossidanti, può rappresentare un eccellente alimento per la veicolazione di ceppi probiotici.

Quale terzo obiettivo è stato valutato il potenziale impiego di farina di castagne per la produzione di prodotti da forno senza glutine. In laboratorio sono stati prodotti impasti acidi maturi adoperando miscele di farine diverse, segnatamente riso, segale, grano saraceno, mais e frumento, e farina di castagna. Le dinamiche microbiche durante la fermentazione sono state monitorate mediante PCR-DGGE mentre il profilo quali-quantitativo dei composti volatili generati negli impasti maturi, è stato monitorato mediante gascromatografia accoppiata alla spettrometria di massa. *Lactobacillus plantarum* e *Pediococcuspentosaceous* dominano gli ecosistemi a lievitazione naturale, indipendentemente dalla miscela di farine impiegata. La RAPD-PCR ha tuttavia rilevato nell'ambito della specie *Lactobacillus plantarum*, una considerevole biodiversità genotipica per i ceppi provenienti da combinazioni di farine senza glutine. Un totale di 59 composti volatili è stato identificato, principalmente alcoli, esteri, acidi, aldeidi e chetoni. L'analisi delle componenti principali dei campioni, all'inizio e al termine della maturazione, ha consentito di ottenere informazioni più dettagliate sui composti volatili ed ha confermato l'influenza della fermentazione sul profilo sensoriale degli impasti acidi.

0.Prefase

According to the FAO, the chestnut worldwide production is estimated in 1.9 million tons. Europe is responsible for about 5% of global production, with relevance for Italy and Portugal (Botondi et al., 2009). Campania region provides 50% of the Italian chestnut crops and, in 1992, one Protected Geographical Indication (PGI) called "Castagna di Montella", was created for chestnuts produced in the Irpinia district.

The chemical composition of chestnut fruits has been recently reviewed (De Vasconcelos et al., 2010) revealing the presence of various nutrients that are important for human health. Chestnuts contain very small amounts of crude fat content that is low insaturated fatty acids and high in monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, which are known for their anticancer effects and for decreasing the risk of cardiovascular diseases and neurological function disorders. Chestnut fruits also contain significant amounts of γ-aminobutyric acid and are a good dietary source of vitamins E, C, B1, B2, B3, pantothenic acid, pyridoxine, and folate and of important mineral macro- (Ca, P, K, Mg and S), and micro- (Fe, Cu, Zn and Mn) elements.

In Italy, chestnuts are mainly consumed roasted, boiled and as ingredient for confectionery preparation. To preserve fruit quality, in Irpinia fresh chestnuts are submitted to traditional water curing post-harvest managements, based on soaking fruits in water typically for about one week. Factors that affect effectiveness of water curing have only been explained partially (Jermini et al., 2006; Botondi et al., 2009). Some studies showed that preservation may result from a light fermentation caused by lactic acid bacteria (LAB) on the fruit. A decrease in pH, likely imputable to a light fermentation caused by lactic acid bacteria, may inhibit the growth of moulds. (Jermini et al., 2006). According to other authors (Bounous et al., 2000), soaking in water may affect permeability of the marron pericarp and, as a result, increases solubility of phenolic compounds of the fruits. This phenomenon, together with lactic acid fermentation, may cause an acid environment thus inducing antifungal activities. Recent studies have also suggested that the antiseptic effect of water curing may depend on an increase in CO_2, acetaldehyde and phenolic compound contents in water, presumably together with an increase in lactic acid content (Botondi et al., 2009). In spite of the importance of this treatment, no attempt to standardize this process through the employment of properly selected microbial strains has never been performed

Moreover, this process produces a lot of wastewater;therefore, the optimization of this treatment would provide an energetic and environment advantage as well as the obtaining of by-products (eg polyphenols) that could be used in the cosmetic and pharmaceutical companies.

Apart from the inefficient post-harvesting and packaging technology, another limit of this chain is due to the scanty efforts carried out to bypass the seasonality of the production through the development and the promotion of innovative foods chestnut-based. Unfortunately, during the last years,the production of the chestnuts from Irpinia was damaged by adverse climatic conditions and the gall wasp (*Drycosmus kuriphilus Yasumatsu*). For this reason the quantity and the quality of the chestnut has been reduced. The development of un-conventional chestnut-based foods would be even a tool to increase in value the imperfect fruits opening up new markets.

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1STATE OF THE ART

1.1 History, culture and botanical characteristics of chestnut tree

Chestnut (*Castanea*), is a genus of eight or nine species of deciduous trees and shrubs in the beech family *Fagaceae*, native of temperate regions of the Northern Hemisphere. The name also refers to the edible nuts they produce. The name *Castanea*is probably derived from the old name for the Sweet Chestnut, either in Latin (*nux castanea*)or in Ancient Greek (*karya kastàneia*). Another possible source of the name is the town of Kastania in Thessaly, Greece, but, more likely, the town took its name from the most common trees growing around it. The most important species are *Castanea* (*C.*) *dentata*, *C. pumila* and *C. chrysophilla* in North America; *C. mollissima* and *C. crenata* in Asia; and *C. sativa* in Europe. Chestnut range from moderate (Chinese) to fast-growing (American and European species) tree. Their mature heights vary from the smallest species of chinkapins, often shrubby, to the giant of past American forests, *C. dentata* that could reach 60 m. In between these extremes may be found the Japanese *C. crenata* (about 10 m), the Chinese *C. mollissima* (about 15 m), and the European *C. sativa* (about 30 m). The native area of *C. sativa* extends from the South of England and the Iberian Peninsula to the closeness of Caspian Sea and North of Morocco and Algeria (Conedera et al., 2004). The European chestnut (*Castanea sativa* Mill.) probably had its origins in the Eastern Mediterranean region over 90 million years ago in the middle Cretaceous, later spreading throughout Europe during the Cenozoic period. Later, between 900 and 700 BC, deliberate chestnut tree cultivation occurred in the Asian regions of the Caspian and Black seas. After this time chestnut cultivation quickly spread to Greece and, later, the Romans discovered the benefits of this nut tree (Adua et al., 1999).

The Chinese, and above all the Japanese chestnuts, are often multi-leadered and wide-spreading, whereas European and, especially American species tend to grow very erect when planted among others, with little tapering of their columnar trunk, which is firmly set and massive. When standing on their own, they spread on the sides and develop broad, rounded, dense crowns at maturity. The two latter's foliage has striking yellow autumn coloring (Conedera et al., 2004). The bark of *C. sativa* is smooth when young, of a vinous maroon or red-brown colour for the American or grey for the European chestnut. With age American species becomes grey and darker, thick and deeply furrowed; the furrows run longitudinally, and tend to twist around the trunk as the tree ages; it sometimes reminds one of a large cable with twisted strands. The leaves are simple, ovate or lanceolate, 10–30 cm long and 4–10 cm wide, with sharply pointed, widely-spaced teeth, with shallow rounded sinuates between (Ferrini et al., 2008). The flowers follow the leaves, appearing in late spring or early summer or onto July. They are arranged in long catkins of two kinds, with both kinds being borne on every tree. Some catkins are made of only male flowers, which mature first. Each flower has eight stamens, or 10 to 12 for *C. mollissima*. The ripe pollen carries a heavy sweet odour that some people find too sweet or unpleasant. Other catkins have these pollen-bearing flowers, but also carry near the twig from which these spring, small clusters of female or fruit-producing flowers. Two or three flowers together form a four-lobed prickly calybium, which ultimately grows completely together to make the brown hull, or husk, covering the fruits. The fruit is contained in a spiny (very sharp) cupule 5–11 cm in diameter, also called "bur" or "burr". The burrs are often paired or clustered on the branch and contain one to seven nuts according to the different species, varieties and cultivars. Around the time the fruits reach maturity, the burrs turn yellow-brown and split open in 2 or 4 sections. They can remain on the tree longer than they hold the fruit, but more often achieve complete opening and release the fruits only after having fallen on the ground; opening is partly due to soil humidity. The chestnut fruit has a pointed end with a small tuft at its tip (called "flame" in Italian), and at the other end, a hilum – a pale brown attachment scar. In many varieties, the fruit is flattened on one or two sides. It has two skins. The first one is a hard outer shiny brown hull or husk, called "pericarpus"; the industry calls this "peel". Underneath the pericarpus is another thinner skin, called "pellicle" or "episperm" (Ferrini et al., 2008). The pellicle closely adheres to the seed itself, following the grooves usually present at the surface of the fruit. These grooves are of variable sizes and depth according to the species and variety. The fruit inside these shows two cotyledons with a creamy-white flesh throughout, except in some varieties which show only one cotyledon, and whose episperm is only slightly or not intruded at all. Usually these varieties have only one large fruit per burr, well rounded (no flat face) and which is called "marron" ("Marron de Lyon" in France, "Marron di Mugello" in Italy, or "Paragon"). The superior fruiting varieties among European chestnuts have good size, sweet taste and easy-to-remove inner skins (Ferrini et al., 2008).

1.2 Chestnut production

According to the FAO (2010), the chestnut tree is mainly grown in Europe and Asia, and chestnut worldwide production is estimated in 1.9 million tons. The Asiatic production of chestnuts covers 70% of world production. China is the leader producer with 445.000 ha, followed by South Korea with 430.000 ha, Turkey with 392.000 ha, Japan with 283.000 ha, and Italy with 275.000 ha (Botondi et al., 2009). Europe is responsible for about 5% of global production, with relevance for Italy and Portugal, corresponding to 2% and 1%, respectively. In 2008, the production of these nuts was 22.000 t for Portugal, 55.000 t for Italy, 6.258 t for France, 15.000 t for Spain, 55.800 t for North and South America, 925.000 t for China, 22.100 t for Japan and 80.000 t for Korea (De Vasconcelos et al., 2010). Europe domestic production and import are reported to be 24.600.000 and 25.200.000 tons, respectively. The estimated *per capita* consumption was found to be 0.4 kg (Roy et al., 2008).

Campania region provides the 50% of the Italian chestnut crops; the Irpinia district, in detail, with about 17.000 tons contribute for about the 40% to the crop of the region and, in 1992, one Protected Geographical Indication (PGI) called "Castagna di Montella", was created for chestnuts produced in this area. Due to its commercial potential, Campania Region government has been granting financial support for the reinforcement of chestnut production.

1.3 Composition of European chestnut (*Castanea sativa* Mill.)

The chemical composition of chestnut fruits have been recently reviewed (De Vasconcelos et al., 2010) revealing the presence of various nutrients that are important for human health. Chestnut fruits are mainly composed of carbohydrates: primarily starch. The free sugar sucrose (saccharose) can be up to one-third of the total sugars. Previous analyses highlighted the presence of various mono- and disaccharides (glucose, fructose, sucrose and maltose) that are very important for the assessment of chestnut fruit commercial quality (Bernàrdez et al., 2004; Mìguelez et al., 2004; De Vasconcelos et al., 2010).

Previous studies on Italian chestnuts to evaluated the specific contents of amylose and amylopectin (Pizzoferrato et al., 1999; Attanasio et al., 2004; Pereira et al 2006) which account for 33 to 67% of the starch content, respectively. Both these forms exert positive health effects, by providing energy for the catabolism of both amylose and amylopectin into glucose and by producing short-chain fatty acids (SCFA) by bacterial catabolism of amylopectin-derived dextrins in the gut (Pizzoferrato et al., 1999; Nichols et al., 2003).

On the other hand, chestnuts contain very small amounts of crude fat content that is low in saturated fatty acids and high in unsaturated fatty acids (Borges et al., 2007). Chestnut fruit dietary contribution to total fat is relatively low. Chestnut fat contains significant levels of monounsaturated fatty acids (MUFA) and PUFA, which are known for their anticancer effects and for decreasing the risk of cardiovascular diseases and neurological function disorders (Hardaman, 2002; Whelan et al., 2006).

The average crude protein content of fresh chestnut fruits was estimated to be 3.5 g 100 g^{-1}, that represents approximately 9.2% of the RDI (Recommended Daily Intake) for females and 7.6% of the RDI for males. The amino acids that constitute the proteins in the diet are of high importance. Amino acids are biologically active and have multiple functions in the body: as sources of energy and as precursors of proteins and various important molecules. Amino acids are divided into essential (those that the human body does not synthesize in sufficient quantities, so they are necessary in the diet) and non-essential (the human body is able to synthesize them in adequate concentrations). There are some reports of measurements for free amino acids in *C. sativa* fruits (Table 1) (De Vasconcelos et al., 2007). The predominant free amino acids in a recent study were aspartic acid, asparagines and glutamic acid (De Vasconcelos et al., 2009).

Table 1 Contents of free amino acids previously reported in chestnut fruits (adapted from De Vasconcelos et al*.,* 2009).

Free amino acids	mg 100 g^{-1} (Fresh Weight)
Essential	
Arginine	0.5-1.7
Isoleucine	0.3-1.4
Leucine	0.3-1.0
Phenylalanine	0.5-1.8
Threonine	3.6-10.0
Trpptophan	0.3-2.2
Valine	0.8-2.7
Non-essential	
Alanine	3.3-10.0
Asparagine	14.9-36.4
Aspartic Acid	2.3-11.3
Glutanine	2.2-7.3
Glutamic Acid	4.6-14.7
Glycine	–
Serine	1.4-3.8
Tyrosine	0.4-1.1

Chestnut fruits also contain significant amounts of γ-aminobutyric acid (GABA, derived from arginine: 50–236 mg 100 g^{-1} DM), which is an important neurotransmitter in the central nervous system (Tixier et al., 1980). Chestnuts are a good dietary source of vitamins E, C, B1, B2, B3, pantothenic acid, pyridoxine, and folate. The average vitamin E content (100 g^{-1}) in fresh chestnut fruits is 1.9 mg, representing, actually 12.7% of the RDI for both females and males. The mean vitamin C level (100 g^{-1}) in fresh chestnut fruits is 15.6 mg, representing 20.8% of the RDI for females and 17.3% for males. In other words, chestnuts can be considered a good dietary source of both vitamins E and C (Korel et al., 2009). The mineral content in chestnut fruits, reveals important macro- (Ca, P, K, Mg and S), and micro- (Fe, Cu, Zn and Mn) elements, with potassium playing a key role within this group (Do Carmo et al., 2010) (Table 2).

Table 2 Content of minerals in chestnut fruit (adapted from De Vasconcelos et al.,2010).

Minerals (mg 100 g^{-1} Dry Matter)					
Ca	P	K	Mg	S	Na
30.8-45.8	99.8-156.4	629.2-811.1	49.4-58.7	19.9-28.4	
Fe	Cu	Zn	Mn	B	Se
3.0-7.1	0.6-0.9	0.6-1.3	1.5-5.1	3.0-3.1	0.4-0.8

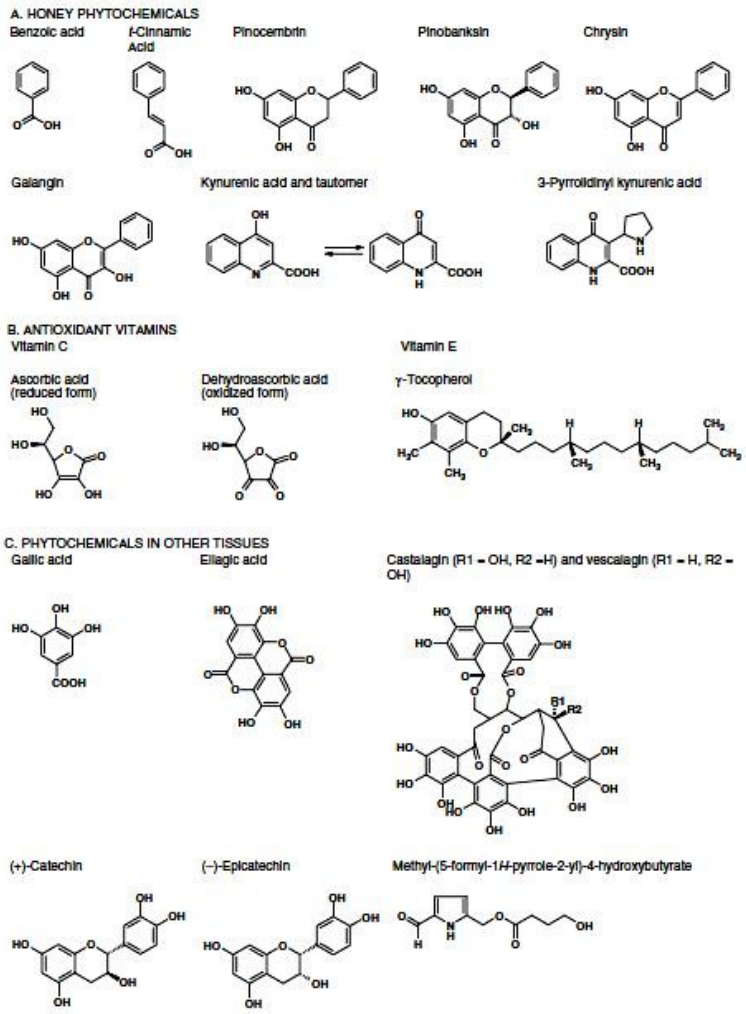
By outcomes regarding nutrients' composition, it is clear that chestnut fruits extracts, and potentially extracts obtained by different parts of the chestnut trees, may have considerable potential as ingredients for novel functional foods development, e.g. chestnut polyphenolic extracts as a natural source of antioxidants and other beneficial compounds such as gallic and ellagic acids and ellagitannins (Figure 1 and Table 3).

Table 3 Content of phenolics and pigments in chestnut fruit (adapted from De Vasconcelos et al., 2010).

Tissue	Phenolics (mg g^{-1} Fresh Weight)					
	Total phenolics		Gallic acid		Ellagic acid	
Wood	-		-		89	
Bark	-		-		0.7-21.6 2.8–18.4[a]	
Leaves	21.5		-		-	
Flowers	48.6		-		-	
Whole shell	26.6–52		-		-	
Outer shell	25.4		-		0.04–0.2 0.7–6.0[a]	
Inner shell	102.6		-		0.03–0.1 0.5–0.8[a]	
Fruit	21.1–36		2.8–9.1 2		7–9.6	
Fruit	0.7		-		0.05 0.05[a]	
Fruit	6.6–18		6.1–25.6		3.8–47.8	
	Pigments (μg g^{-1} Fresh Weight)					
	Lutein	*β*-Caroten	Total minor carotenoids	Lutein esters	Total carotenoids	Total chlorophylls
Wood	0.2	0.7	-		-	-
Leaves	-	-	-		14.7–22.6[b]	78.2–119.6[b]
Fruit	0.1[c]	0.1	-		-	-
Fruit	0.1–0	0.01–0	0.02-0	0 0.1–0	0.23–1.30	

[a]The first values are for non-hydrolyzed content, the second for acid hydrolyzed.
[b]Values are presented as mg cm^{-2}.
[c] Lutein plus zeaxanthin.

Figure 1 Phytochemicals and vitamins from various tissues of the *Castanea Sativa* tree (De Vasconcelos *et al*., 2010).



Dietary polyphenols receive considerable interest for their presumed role in the prevention of various degenerative human diseases such as cancers and cardiovascular diseases (Rice-Evans et al., 1997; Scalbert et al., 2002). Despite their beneficial effects on human health, chestnuts and other fruit and vegetables may represent potential carriers of toxic substances such as xenobiotic trace elements that are deleterious for plant, animal and human systems (Patra et al., 2004). Within xenobiotic elements, As, Cd, Hg, Pb and Ni are the most important to consider in terms of food chain contamination (Peralta-Videa et al., 2009). Soils are usually enriched in these elements through the agricultural, industrial or urban activities of man.
Although some studies have been carried out on the nutritional composition of European chestnuts, data concerning the Italian varieties and about the possible changes in secondary metabolites and mineral composition after different cooking procedures or after curing are still scarce or incomplete and variable (Attanasio et al., 2004; Bellini et al., 2006; Neri et al., 2010; Sacchetti & Pinnavaia, 2005). Nazzaro and coworkers (2011) assessed total and specific polyphenols content, antioxidant activity and mineral and trace elements, in the same local chestnut ecotype from Montella (Avellino, Italy) considered in the present work. Moreover, authors performed analyses on raw and cured chestnuts, as well as on chestnuts subjected to different cooking methods, in order to verify the qualitative changes induced by such treatments. Their study indicated that cooking processes, as well as the curing treatment, may affect the chemical composition and the biological activity of chestnuts. In particular, the curing treatment induced a decrease in the content of all the investigated parameters. On the other side, roasting proved to be the method that best preserves minerals, including the trace metals, as well as the total and specific polyphenolic content. In all cases, authors concluded that it is essential that the fruits grow in an uncontaminated environment (Nazzaro et al., 2011).

1.4 Chestnut uses and industrial processing

Fresh chestnut fruits are rarely consumed raw. They are processed in several ways, at home or on an industrial scale to improve organoleptic properties (aroma, flavour, texture), digestibility of the fruits (i.e. making nutrients more bioavailable), and shelf-life of the different chestnut-based products. After harvest, all cultivars are ready for consumption; however, certain cultivars are more appreciated because of their organoleptic properties, although calibre and maturation

time are factors to be considered as well.

For commercial purposes, chestnuts are often classified by size, being the smallest fruits used in the industry and the biggest fruits destined to the fresh fruit market (Bounous et al., 2002). Because of the seasonal character of the production and the short shelf life of the chestnut nut (Bergognoux, 1978; Tan et al,. 2007) due its high metabolic activity, several preservation methods have been proposed to avoid weight loss and contamination by insects, microorganisms or enzymatic reactions (such as starch degradation), which cause a great decrease of quality (Anelli et al., 1984; Breisch 1993). In the past, as a preservation method, chestnuts used to be dried by air at room temperature. In such form, namely as a hard and yellowish product, chestnuts could be consumed throughout the year. Recently, the corresponding drying kinetics studies at different experimental conditions have been published, evaluating simultaneously some parameters of quality in dried product (Koyuncu et al., 2004; Moreira et al., 2005; Guiné & Fernandes, 2006). Because of the sensitivity of chestnuts to high temperatures, sterilization (the method used for preservation of quality characteristics of other food such as carrot, turnip top, etc.) is made more problematic by starch and sugar degradation (Pinnavaia et al., 2003). Nevertheless, other methods for chestnut preservation are available. Thermo-hydrotherapy, the immersion of fruits into water at 50°C for 45 min followed by drying, prolongs the storage time up to 3–4 months; hydrotherapy , the immersion into water at 18–20°C for 4–7 days followed by drying, allow to prolong preservation up to 5–6 months; drying, the reduction of the fruit moisture content, is used for small fruits to be peeled and transformed into flour; and preservation with artificial respiration in cold chambers at 0 to 2°C and 90–95% of relative humidity, allows a preservation from 3 to 4 months.On the other hand, preservation in modified atmospheres in order to keep the product characteristics unchanged offers two alternatives: storage at 1°C with CO_2 (20%),O_2 (2%) and 95% relative humidity provides a preservation period of 4 to 6 months; storage at high levels of CO_2 (45–50%) followed by 4–5 days in contact with air, preserves the chestnut under acceptable conditions for one month; freezing at -40°C for 12 h, and then at –20°C at 80–90% of relative humidity, makes possible to preserve the chestnuts for one year and it's usually used for high-quality chestnuts, such as marrons (Jermini et al., 2006).

Frozen chestnuts are one of the most common forms for this food, and are available all year round to consumers. According to recent surveys, industrial processes have variable effects on chestnut fruit nutrients. The positive effects on the fruits include the extension of the shelf life and the increase in neutral detergent fibre (NDF) (12.7-19.9 and 16.3-18.2 g 100 g^{-1} DM), total phenolics (6.6–12.0 and 10.6–16.9 mg g^{-1} fresh weight), Threonine (4.9–6.8 and 3.6–7.9 mg 100 g^{-1} FW) and Alanine (3.3–6.5 and 4.0–5.7 mg 100 g^{-1} FW) when passing from stage A (fresh) to stage D (frozen) in 2006 and 2007, respectively, as well as in δ-tocopherol (0.3–0.4 µg g^{-1} FW in 2006) and δ-tocopherol (25.2–27.3 µg g^{-1} FW in 2007).The negative effects were reductions in the levels of total starch (53.8–51.3 and 54.9–51.0 g 100 g^{-1} DM), Glutamic Acid (14.7–9.2 and 13.9–10.9mg 100 g^{-1} FW) and ascorbic acid (6.4–5.4 and 7.2–5.1 mg 100 g^{-1} FW) in 2006 and 2007, respectively, and also of fat (2.9–2.5 g 100 g^{-1} DM in 2007) (De Vasconcelos et al., 2009; De Vasconcelos et al., 2010).

In frozen chestnut products, the losses related to dehydration are drastically decreased, conferring to the fruits a long-term (out-of-season) availability and compositional stability. As part of industrial processing Hwang et al. (2001) previously analyzed the physicochemical factors related to the automatic pellicle removal in 14 Korean chestnut (*C. crenata*) varieties. These authors stated the existence of a negative correlation between the tannin content of the integument and the peeling ratio. The kernels used for the production of sweet chestnuts have to be, as previously described, in optimal condition after storage and have a good caliber. Some chestnut fruits are infiltrated with increasing concentrations of sugar solutions and conserved in flasks or metallic protected bags. Others can be added to bottles containing alcoholic beverages, allowing the sugar in the fruit to sweeten the liquor for a period of 6–12 months, e.g. brandies, cognacs or digestive liquors.

In France, Italy, Switzerland and Spain the most appreciated processed chestnut fruits are the so called 'marrons glacées'. For the preparation of these confectioneries, chestnuts are submerged in a sugar-rich solution and then covered with glucose. The fruits are then cooked in an oven at 300°C for 1-2 minutes to crystallize the sugar (López et al., 2004). Freeze-dried chocolate-coated Chinese chestnut (*C. mollissima*) fruits have been recently produced and evaluated by a nutritional and microbiological point of view (Gounga et al., 2008). According to authors, this type of processing allows the decrease of microbial loads and therefore the extension of the shelf life.

Some studies have been performed to evaluate the enzymatic hydrolysis of chestnut products (steamed fruit and purées) with mixtures of α-amylase and amyloglucosidase, in order to develop spirits and glucose syrups by distillation of the produced glucose (López et al., 2004; López et al., 2005). Other authors evaluated the potential of the fruits for the production of chestnut-based chips (Di Monaco et al., 2010).

Other recent preservation methods in development are lyophilization, which results in high-quality products but with high costs, and the combined methods by means of osmotic dehydration and convective air-drying (Chenlo et al.2006a,b).

In Italy, the most diffuse treatment for chestnuts preservation is the water curing. Water curing was known in the past as "novena", since chestnut fruits were kept for nine days in the water. The objective of this method is to lessen the development of fungi during storage and, at the same time, to favor the killing of the worms. The efficiency of the method depends on partial lactic and alcoholic fermentation that takes place during the curing process, which reduces pH and allows diffusion of phenols from the episperm into the flesh (Botondi et al., 2009). Soaking in water may affect permeability of the marron pericarp and, as a result, increases the solubility of phenolic compounds of the fruit. This phenomenon, together with lactic acid fermentation, may cause an acid environment inducing antifungal activities. An antiseptic effect of water curing may depend on an increase in CO_2, acetaldehyde and phenolic compound contents in water, presumably together with an increase in lactic acid content (Botondi et al., 2009).

The following steps are important for a good water curing treatment:

- keep chestnuts into water, better if at 15°C, for 3 to 7 days: The start of fermentation is advised by bubbling; pH of the water goes drops of around one unit;7 days guarantee the worms killing;
- remove chestnuts from the water, spread chestnuts in a layer of 5-10 cm and let them to wipe very well in air stream;
- rotate chestnuts preferably every day.

Storage is one of the major problems in chestnut postharvest process, since the product is very susceptible to fungi contamination Chestnuts are living tissue and, thus, perishable; the only way to slow down their living activity, such as respiration, is to reduce, first of all, the temperature (Mencarelli, 2001). As a matter of fact, chestnuts continue to respire after harvest using starch as a substrate. According to Migliorini et al. (2010), this phenomenon seems to decrease during water curing. As matter of fact, authors recorded constant starch contents in the endosperm during water curing. Moreover, according to Migliorini et al. (2010), hydration of fruit during curing resulted in decreased moisture loss during storage and hence, longer shelf-life of the product. Moreover, according to data provided by these authors, water curing has an inhibiting effect on fruit infecting fungi (i.e. *Ciboria batschiana*, agent of black rot). A phenomenological hypothesis to explain the above-mentioned preservation effects may be proposed as an improvement on the literature data on curing (Bounous, 2002; Botondi et al., 2009). Curing includes a washing activity that likely induces a considerable removal of the surface microflora. At the same time, it also includes a sequence of combined physic-chemical phenomena, which are able to affect both structure and biological contamination of marrons. Surface layers of the fruit are hydrated by mass convection phenomena, resulting in diffusion of water from pericarp to endosperm. Hydration causes mass (i.e. phenolic compounds) transfer by diffusion from the episperm to the pericarp. The increase in phenolic compounds concentration in pericarp and, as a result in curing waters, results in decreased microbial counts. This decline did not seem to be affected by a drop in pH, caused by lactic acid fermentation in curing water. During storage, because of the oxidation, a decrease in phenolic compounds concentration has been reported (Botondi et al., 2009).

Aspergillus sp., *Penicillium* sp., *Trichotecium* sp., *Fusarium* sp., *Rhizopus* sp., *Trichoderma* sp., and *Cicoria batschiana* are the most common moulds isolated from chestnut fruits by Migliorini et al. (2010). According to these authors, soaking chestnuts resulted in a lower total fungal microflora in the pericarp. This may related to both the washing action and the inhibiting role of phenolic compounds, which diffused to the marron surface (Migliorini et al., 2010). Malic and lactic acid, as well as sugar and ethanol contents were found in traces and did not change significantly (Migliorini et al., 2010). Water curing of marrons produces a lot of carbon dioxide as a result of aerobic and anaerobic metabolism, but no data have been presently provided to justify this production (Botondi et al., 2009). According to Botondi et al. (2009), the lowering of waters pH may activate the fermentation by stimulating the lactic dehydrogenase, and consequently the pyruvate decarboxylase. An increase in CO_2, acetaldehyde and polyphenols, together with a presumed increase in lactic acid could be the reason for the antiseptic effect of water curing. Yet, this can happen within certain time limits and with specific water/marrons ratios. Moreover, according to data provided by Botondi et al. (2009), polyphenols increase until day 5^{th} of water curing above all when water is artificially acidified and then decrease on day 7^{th}. Waters acidity seems able to stimulate the anaerobic metabolism, increasing the acetaldehyde concentration during curing and even the polyphenol content above all in the water/marron 1:1 ratio, but this response appears to be a stress response, which does not guarantee the antiseptic effect during the subsequent air storage where ethanol increase was observed(Botondi et al., 2009). Moreover, according to Botondi et al. (2009), chestnuts must be immersed in water for a maximum of five days to maintain cells survival and a water/marrons ratio of 1:1 is advised.

1.5 Chestnut as a functional food

According to the International Life Science Institute (ILSI), a food can be considered functional if it can demonstrate satisfactorily that it has a beneficial effect on one, or several specific functions in the organism, beyond the normal nutritional effects that improve the state of health and well-being, or reduce the risk of a disease.

Fermented foods containing probiotic microorganisms are generally considered as functional foods. Probiotic is a relatively new word meaning ‘‘for life’’ and it is generally used to name the bacteria associated with the beneficial effects for the humans and animals. The term probiotic was technically defined by an Expert Committee as ‘‘live microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition’’ (FAO/WHO, 2001). Many nutritional and therapeutic benefits have been attributed to probiotic microorganisms, including metabolism of lactose, control of gastrointestinal infection, suppression of cancer, reduction of serum cholesterol, immune stimulation, and increased digestibility of foods (Collins et al., 1998).

The therapeutic potential of probiotics is dependent upon a number of factors, but most important are their survival during manufacture, storage, transit through the gastrointestinal tract, and the ability to proliferate in the large intestine (Tannock, 1998). Selection of suitable strains must account for these factors (Saarela et al., 2000). The main challenges presented to probiotics, during their passage through the gastrointestinal tract (GIT), are the acidic gastric secretions of the stomach, and the bile salts released into the duodenum (Bezkorovainy, 2001). Several researchers studied the survival of microorganisms in the presence of bile salts and at low pH, and it has become clear that the survival of probiotics, both *in vitro* and *in vivo*, is strongly influenced by the food used for their delivery (Charteris et al., 1998; Dunne et al., 2001). Buffering capacity, pH, as well as physical and chemical characteristics of the food carrier have all been demonstrated to be significant factors. Foods used for the delivery of probiotics are usually dairy fermented foods (Gomes & Malcata, 1999), such as yoghurt, fermented milk, ice cream and cheeses. One of the main setbacks of consuming dairy products by those with deficiency in the enzyme β-galactosidase is leading to abdominal cramping, bloating, diarrhoea and nausea. For this reason an increasing

interest is currently found for different food matrices which may offer a number of advantages (Prado et al., 2008). The good adaptation of lactic acid bacteria in chestnut-based media suggests that the utilization of potentially probiotic strains, as starter culture in this substrate, would produce a fermented food with defined and consistent characteristics and possibly health-promoting properties (Blaiotta et al., 2012).

From the various composition and health studies it is clear that chestnut fruits, and potentially other extracts from chestnut, have considerable potential as functional foods or as food ingredients, e.g. chestnut polyphenolic extracts as a natural source of antioxidants and other beneficial compounds such as gallic and ellagic acids and the ellagitannins. It has previously been shown that many ellagitannins, including castalagin and vescalagin, have potent antitumor, antioxidant, antimicrobial and antimalarial properties (Kasiwada et al., 1992; Reddy et al., 2007).

In recent times, there has been a growing interest in the use of chestnut flour for the production of leavened bakery goods. Chestnut flour may be used in gluten-free flour breads due to its nutritional and health benefits. Chestnut flour contains high quality proteins with essential amino acids (4–7%), relatively high amount of sugar (20–32%), starch (50–60%), dietary fiber (4–10%), and low amount of fat (2–4%). It also contains vitamin E, vitamin B group, potassium, phosphorous, and magnesium (Chenlo et al., 2007). Since most of the gluten-free products do not contain sufficient amount of vitamin B, iron, folate, and dietary fiber (Moroni et al., 2009), it may be advantageous to use chestnut flour due to its nutritional value.

Baking of gluten-free flours is a challenge, due to the lack of gluten proteins, as gluten is a protein which possesses structure-forming ability that affects elastic properties of dough and contributes to the overall appearance and crumb structure of many baked products. Therefore, the removal of gluten in gluten-free formulation is a very demanding task, resulting in often low quality, poor mouthfeel and low-flavour products (Moroni et al., 2009).

In order to improve bread performance, sourdough could be used. The use of the sourdough process as a means of leavening is one of the oldest biotechnological processes in cereal food production. Sourdough is a mixture of mainly cereal flour and water, which is made metabolically active by a heterogeneous population of LAB and yeasts, either by spontaneous fermentation or by fermentation initiated through the addition of a sourdough starter culture, whether or not involving back slopping (De Vuyst et al., 2009). The partial abandonment of traditional bread with ‘‘natural yeast’’, for bread made from industrial yeast, is mainly due to the possibility of simplifying the process, because the traditional system is generally more time-consuming and difficult to maintain stable over time.

Currently, for the recognized and documented scientifically valuable characteristics of sourdough-based bakery products, there is, worldwide, a renewed interest in this type of processing. Most rheological, sensory, nutritional advantages, and the recorded extended shelf-life, mainly derive from microbial metabolic activities, such as fermentation, proteolysis, synthesis of volatile compounds and antifungal activity (Hammes et al., 1998). The positive contribution of sourdough could be exploited for the production of high quality gluten-free bread from various gluten-free cereals and pseudocereals, with reduced need for expensive additives (Moroni et al., 2009).

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2 AIMS OF THE STUDY

2.1 Optimization of water curing

Chestnuts are very perishable fruits, whose quality may be compromised during postharvest handling (Maresi et al., 2013). Damage can be caused both by insects and fungi (Wells & Payne, 1980). Water curing, a commonly used postharvest method, is based on soaking fruits in water typically for about one week (Botondi et al., 2009). Factors that affect effectiveness of water curing have only been explained partially. A decrease in pH, likely imputable to a light fermentation caused by LAB, may inhibit the growth of moulds (Botondi et al., 2009). In the light of the above considerations, the aim of this study was firstly to evaluate the evolution of microbial population along the *Curatura* process and, secondly, to assess the possibility of using LAB strains to inhibit the growth of spoiling fungi. In addition, the environmental impact of the water curing may be decreased by performing a sort of backsloping process. In detail, three different trials will be proposed: *i)* water curing with adjunct of strain *Lactobacillus* (*Lb.*) *pentosus* OM13 characterized by antifungal activity; *ii)* traditional water curing with tap water; and *iii)* water curing with residual water recovered by previous curing treatments. Each trial will be performed on a laboratory scale as well as on a farm scale. The evolution of total microflora, lactic acid bacteria, yeasts and moulds will be followed by means of a polyphasic approach based on conventional techniques and PCR-DGGE-based methods.

2.2 Production of novel and functional foods by fermentation of chestnut-based matrices

The objective was to develop new chestnut-based foods by means of alcoholic and lactic fermentation, in order to seasonally adjust the offer and to use the surplus of undersized production, providing, at the same time, a response to the growing demand for healthy and environmentally friendly products. By alcoholic fermentation, a chestnut-flavored beer will be produced, while by using LAB cultures a functional chestnut puree will be developed. Broken dried chestnuts, a product usually employed for flour production or for animal feeding, will be used to produce a functional beverage lactose-free and with reduced fat content. In detail, chestnut purees will be use as medium for the growth of selected LAB strains. Fermented purees will be characterized by a technological and sensorial point of view, while the LAB strains employed for the diverse fermentations, will be tested for their probiotic potential by conventional *in vitro* tests. In other words, the goal will be the development of a new concept food able to join the functional properties of the chestnut fruits with the benefits provided by the ingestion of probiotic strains on human health.

2.3Chestnut employment for the development of functional and probiotic foods

The main challenges to probiotics, during their passage through the GIT are the acidic gastric secretions of the stomach, and the bile salts released into the duodenum (Bezkorovainy, 2001). The survival of the strains, in this phase, is strongly influenced by the food used for their delivery (Charteris et al., 1998; Dunne et al., 2001).

Actually, chestnut appears as a functional fruit and, moreover, due to the presence of non-digestible components of the matrix, chestnut might also serve as prebiotics. However, due to the complexity of chestnut composition, a systematic approach is needed in order to identify the factors which may enhance the growth and the survival of probiotic strains, both *in vitro* and *in vivo*.

The overall aim was, in this case, to evaluate chestnut-based substrates as probiotic carriers by examining the effect of indigestible chestnut fiber and of chestnut extract on the viability of selected LAB strains.

Moreover, the effect of chestnut extract on the viability of selected LAB cultures the after spray drying will be examined as well. Specifically, two strains will be used to enrich an anhydrous basis for chestnut mousse. Probiotic *Lb. rhamnosus* LbGG (ATCC 53103) and RBM526 isolated from Parmigiano Reggiano cheese, and selected for the high survival under GIT conditions will be incorporated as powders to the mousse. Shelf life and sensorial profile of the chestnut mousses added of lactobacilli dried in chestnut extract will be evaluated as well.

2.4 Use of chestnut flour for the improvements of bakery goods

Currently, for the recognised and documented scientifically valuable characteristics of sourdough-based bakery products, there is, worldwide, a renewed interest in this type of processing. Most rheological, sensory, nutritional advantages, and the recorded extended shelf-life, mainly derive from microbial metabolic activities, such as fermentation, proteolysis, synthesis of volatile compounds and antifungal activity (Hammes & Ganzle, 1998).

The positive contribution of sourdough could be exploited for the production of high quality gluten-free (GF) bread from various GF cereals and pseudocereals, with reduced need for expensive additives (Moroni et al., 2009).

In recent times, there has been a growing interest in the use of chestnut flour for the production of leavened bakery goods. Chestnut flour may be used in GF flour breads due to its nutritional and health benefits. In the light of the above considerations, spontaneously fermented chestnut-flour-based sourdoughs, propagated under technological conditions similar to those used for the production of industrial wheat and rye sourdough will be formulated. Fermentations will be monitored by a polyphasic approach including conventional and culture-independent PCR-DGGE-based methods. By mature sourdoughs, LAB and yeast strains will be isolated, purified, identified and characterized by a genotypic and biochemical point of view. Selected strains will be adopted to develop a sort of adjunct culture able to mimic the fermentation by sourdough employment

The impact of fermentation on volatile organic compounds formation during sourdoughs maturation by means of gas chromatography coupled to mass spectrometry (GC/MS) will be evaluated as well.

2.5 References

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3 RESULTS AND DISCUSSION

3.1 Optimization of water curing for the preservation of chestnuts (*Castanea Sativa* mill.) and evaluation of microbial dynamics during process by means of a polyphasic approach

Chestnuts are typical seasonal fruit that maintain their optimal commercial quality, turgescence and health for only a comparatively brief period. Chestnuts possess relatively high moisture content and dry out rapidly if compared with other edible nuts. Attacks by insect larvae (e.g., *Cydia splendana Hb., Cydia fagiglandana Zell.* and *Curculio elephans Gyll.*) starts while fruits are still on the trees and are well known to be detrimental to the harvesting of healthy chestnut fruits (Maresi et al., 2013). Fungal infections often start in the larval galleries of insects, and nuts become infected on the ground before picking (Wells & Payne, 1980). Several fungi are capable of colonizing nuts and causing damage*: Phoma endogena* Speg., *Phomopsis endogena* Speg. *Cif.* and *Gnomoniopsis* sp. have been described as agents of brown rot, *Sclerotinia pseudotuberosa* Rehm (syn. *Ciboria batschiana* Zopf) can cause black rot, while *Penicillium* spp. and *Penicillium crustaceum* L. Fr. produce greenish moulds (Maresi et al., 2013). At early infection stages, it is not easy to differentiate slightly mouldy or parasitized nuts from good ones until they are processed or consumed (Wells & Payne, 1980). As a consequence, various nut-treatment techniques have been developed. The most common are hydrotherapy (cold bath), or *Curatura*, and thermo-hydrotherapy (warm bath), which was developed in. Italy during the 1930s (Voglino, 1928). The cold-bath treatment has the advantage of not requiring any special equipment and of maximizing the weight of the fruit, but it has several disadvantages: it takes up a large space, immobilizes nuts for almost 10 days, makes the treated fruits lose the natural lustre and cannot guarantee the total elimination of *Curculio elephas* larvae (Jermini et al., 2006). In Italy, the most common chestnut treatment is the cold water curing, known in the past as "novena", since fruits were usually kept in water for nine days (Botondi et al., 2009). Determining factors that affect effectiveness of water curing have only been explained partially (Botondi et al., 2009; Jermini et al., 2006; Migliorini et al., 2010). The efficiency of the method depends on partial lactic and alcoholic fermentation that takes place during the curing process, which reduces pH and allows diffusion of phenols from the episperm into the flesh (Botondi et al., 2009). More generally, antifungal effect of water curing can be related to an increase in CO_2, acetaldehyde and phenolic compound contents in water, presumably together with an increase in lactic acid content (Botondi et al., 2009).

3.2 Materials and methods

3.2.1 Microorganisms and culture conditions

LAB strains used in this study were as follows: three *Lb.rhamnosus* (VT1, RBM526, RBT739) isolated from Parmigiano reggiano cheese (Blaiotta et al., 2012), two *Lb. casei* (491, 496) isolated from Provolone del Monaco cheese (Aponte et al., 2008), nine *Lb. pentosus* (OM13, OM14, OM24, OM35, OM50, OM52, OM53, OM62, OM64) and five *Lb.coryniformis* (OM58, OM59, OM60, OM68, OM69) isolated from table olives (Aponte et al., 2012), *Lb. rhamnosus* GG (ATCC 53103), plus 24 unidentified LAB strains isolated from water kefir grains (1A, 3A, 4AP, 4AM) and probiotic drinks (1B, 2B, 3BP, 3BM, 4B, 5BM, 1C, 1DP, 1DM, 2D, 1E, 2E, 3EP, 3EM) and tablets (4E, 5EP, 5EG, 1S, 2S).

3.2.2 Moulds isolation and identification

For moulds' isolation, fruits were randomly taken during a conventional water curing process (n. 10) and along storage (n. 2) in a farm ("Malerba Castagne") located in Montella (Avellino district, Campania Region, Italy). In detail, ten shelled fruits were diluted with 0.9% NaCl (ratio 1:9), homogenized with a Stomacher Lab-Blender 400 (Seward Medical, London, United Kingdom) for 2 min, serially diluted and plated in triplicate on Dichloran Rose Bengal Chloramphenicol (DRBC) agar base with the addition of Chloramphenicol Selective Supplement (Oxoid, Basingstoke, United Kingdom). After incubation in the dark at 25°C for 3-4 days, twelve moulds were randomly isolated by countable agar plates. Axenic cultures were derived by spores repeated streaking. Spores were removed with a disposable sterile plastic loop, suspended in semi-solid agar and then spread across Malt Extract agar plates (Oxoid). Isolates were identified by sequencing of regions D1/D2 of 26S rDNA or ITS (ITS1-5.8S-ITS2 rDNA region). In detail, DNA was extracted according to protocol proposed by Ciardo et al. (2007). Approximately 2 to 4 cm^2 of the mycelium of fungal culture was added to 200 µL InstaGene™matrix (Bio-Rad, Reinach BL, Switzerland) and incubated for at least 3 h at 56°C. After 10 min at 95°C followed by centrifugation at 16000 *g* for 3 min, the supernatant was used for amplification. In case of PCR failure, a newly collected fungal mycelium was digested at 37°C for 2 h with 30 U of Lyticase (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) in 200 µL of digestion buffer (50 mmol L^{-1} Tris–HCl, 1 mmol L^{-1} EDTA, pH 8.0). Alkaline lysis was then performed by addition of 10 µL of 1 mol L^{-1} NaOH and 10 µL of 10% sodium dodecyl sulphate in incubation at 95°C for 10 min. After neutralization with 10 µL of 1 mol L^{-1} HCl, DNA was purified with the QIAamp DNA blood mini kit (Qiagen, Milan, Italy) according to the instructions of the manufacturer. The DNA was finally eluted in 100 µL of EB buffer.

D1-D2 and ITS regions were amplified by using primers and procedures described by Kurtzman & Robnett (1998) and White et al. (1990), respectively. PCR amplicons were run on TBE agarose gels; plugs were excised from agarose and purified by QIAquick Gel extraction kit (Qiagen) according to the manufacturer's instructions. Sequencing was carried on by Gene Chron (Roma, Italy), while research for DNA similarity was performed with the National Centre of Biotechnology Information GenBank (Altschul et al., 1997).

3.2.3 Screening assay for antifungal activities

All LAB strains were screened for antifungal activity by "Spot on lawn assay", according to procedure described by Shillinger and Villareal (2010). Briefly, strains to be tested were spot inoculated onto MRS agar plates (De Man, Rogosa and Sharpe medium, Oxoid) and allowed to grow at 30°C for up to 3 days in aerobic as well as anaerobic conditions (Anaerogen Kit, Oxoid). The plates were then overlaid with malt extract (ME) agar (0.7%, 9 mL) containing about 10^5 spores of each mould per mL. After incubation in the dark, for up to 5 days at 25°C, plates were examined for the formation of inhibition zones around the bacterial colonies. Tests were repeated by cultivating LAB strains in MRS with reduced glucose content (0.2%) and without acetate and with both modifications. LAB loads reached in the different media were previously estimated by drop method (Collins et al., 1989) onto MRS agar plates.

Eleven promising LAB strains, selected according to the obtained results, were further tested by "Mould agar spot assay" according to Shillinger and Villareal (2010). For the preparation of culture supernatants, LAB strains were grown in MRS broth at 30°C. Cultures were centrifuged at 8160 *g* for 10 min and filtered by AcroDisc millipore (0.22 µm). Supernatants were even obtained by strains grown in MRS broth without acetate and MRS with reduced glucose content (0.2%). Then, filter-sterilized culture supernatants were mixed with 1.5-fold concentrated ME agar at 55°C and poured into small Petri dishes (5.5 cm). 10 µL of the spore suspension (10^5 mL^{-1}) were dropped on the agar. The diameter of the mould colonies was measured by means of an Image Analysis protocol. Plates were incubated for 5 days at 25°C and then were photographed with a digital camera Olympus® C-7070Wide ZOOM camera (Olympus, Milan, Italy). Images were processed by Image tool software: Image Pro Plus 6.1 for Windows® (Media Cybernetics Inc.). The images were recorded in true colour at 512 x 512 pixel resolution and converted to a 256 level grey scale. Calibration of pixel measurements to millimetre based units was accomplished by digitizing a centimetre with precise and constant dimensions under the same conditions as those used when sample images were acquired. The diameter of each mould colony was determined by image analyser and compared with the control in which the supernatant was replaced by MRS medium, MRS at pH 4.00 (HCl addition) and MRS with 144 mmol L^{-1} of L-lactate (pH 4.00). Each value represents the mean of three independent measurements. pH of LAB cultures employed for supernatants production was measured using a pH meter (Seven Easy pH S20, Mettler-Toledo, Giessen, Germany), while viable counts were determined by drop counting (Collins et al., 1989) onto MRS agar. The identification of three interesting unidentified LAB strains was obtained by 16S rDNA sequencing, according to the following procedure. To extract DNA, LAB cultures were transferred to MRS broth, and incubated at 30°C for 48 hours. 2mL of broth cultures were used for the extraction by performing the protocol firstly described by Aponte et al (2012). 16S rDNA sequencing was carried on according to procedure described by Aponte et al. (2008) and using the forward primers (FD1) designed by Weinsburg et al. (199). DNA similarity was determined by BLAST search tool within the National Centre of Biotechnology Information, GenBank (Altschul et al., 1997).

3.2.4 Evaluation of ability of selected lactobacilli to grow in water curing environment

Overnight cultures in MRS broth of selected *Lactobacillus* spp. strains were centrifuged and resuspended in sterile saline solution. Cell suspensions were used to inoculate (about 10^6 mL^{-1}) 5 mL of water obtained by a curing process at the second day performed in farm ("Malerba Castagne" Montella) and sterilized by filtration (0.22 µm). After 48 hours of incubation at room temperature, pH and CFU mL^{-1} by drop method (Collins et al., 1989) were evaluated.

3.2.5 Experimental plan

Chestnuts from the cultivar "Castagna di Montella" were harvested in the area surrounding Montella, on Malerba's organic farm (650 m altitude), and submitted within 48 h to water curing process according to a 1:2 chestnut/water ratio. Firstly, processes were performed in laboratory by placing on containers lots of approximately 5 kg of chestnuts. The sample contained 70.0±1.37 fruit kg^{-1}, on average, belonging to the second commercial category. The vessels were kept in a cold room at a temperature of 18±1°C. Following the treatment, chestnuts were collected and left to dry in perforated polypropylene trays (36 x 26 x 12 cm) at room temperature with daily turning. In detail, three different trials were carried on: A. Curing in tap potable water to which the anti-fungal strain *Lb. pentosus* OM13 was inoculated at the second day of process; B. Curing in normal tap potable water (control process); C. Curing in water that had been recovered from a previous curing process performed in the farm "Malerba Castagne". Each trial was performed in triplicate. The same set of trials just described was even performed on a farm scale, during the same season of production, with batches of chestnuts of about 200 Kg (Farm "Malerba Castagne"). In both cases, samples of water were daily monitored by determination of pH, total microflora on PCA (Plate Count Agar), yeasts and moulds on DRBC agar, LAB on MRS plus cycloheximide (0.17 g L^{-1}; Sigma-Aldrich), *Enterobacteriaceae* on VRBGA (Violet Red Bile Glucose agar) and bacterial spores on PCA after heat treatment of cell suspensions at 80°C for 10 min. All media and supplements used were provided by Oxoid. During the drying of chestnuts cured on a laboratory scale, the following parameters were evaluated: yeasts and moulds on DRBC, weight loss, pH, and titratable acidity. Weight loss was reported as a percentage variation with respect to the weight of the whole vessel at the beginning of testing.

3.2.6 Population dynamics during curing experiments

3.2.6.1 DNA extraction from bulk cells

Samples (50 mL), daily collected from the time zero up to the end of both kinds of processes (pilot and farm scale), were filtered by membrane filtration vacuum systems (AcroDisc Millipore). Membranes (0.22 µm) were put on plates of PCA

for evaluation of total microflora and MRS agar plus cycloheximide (0.17 g L^{-1}) for LAB. After incubation at 30°C for 48 hours, colonies grown on filters were aseptically removed, resuspended in a suitable volume of quarter strength Ringer's solution (Oxoid) and stored at -20°C until use. DNA was extracted according to the protocol developed by Aponte et al. (2012). In detail, cells were grown in 10 mL of MRS up to the exponential phase, washed in STE (100 mM NaCl, 10 mM TriseHCl, 1 mM EDTA, pH 8.00) and suspended in 250 mL of lysis solution consisting of ET (50 mM Tris HCl, 5 mM EDTA, pH 8.00) containing 1.6 mg mL^{-1} of lysozyme, 40 U mL^{-1} of mutanolysin and 0.4 mg mL^{-1} RNase. After overnight incubation at 37°C, 25 mL of 25% (v/v) sodium dodecyl sulphate and 2 mL of pronase (20 mg mL^{-1}) were added, and incubation was allowed to proceed at 37°C for 1 h. A quantity of sodium acetate (5 M) equal to the volume of the water phase was added and, after cooling at 0°C, DNA was precipitated with 0.7 volume of isopropanol. After centrifugation (14,000 *g* for 10 min at 4° C), the pellet was washed with 70% (v/v) ethanol and left to dry. Extracted DNA was dissolved in 50 mL of rehydratation buffer (Promega, Madison, WI, USA) and treated at 55°C for 1 h. All reagents, where not differently detailed, were provided by Sigma-Aldrich.

3.2.6.2 PCR amplification of variable region V3 of 16S rDNA

In order to increase the amount of amplicons of hypervariable region V3 within 16S rDNA, nested PCRs were performed. In other words, the entire 16S rRNA was amplified by means of universal primers (FD1 and RD1) designed by Weinsburg et al. (1991) and used as template for PCR amplification of hypervariable regions V3. Conditions, temperature profile and reaction mixture, for 16S rDNA amplifications were the same reported by Aponte et al. (2013). The primers V3f and V3r (Muyzer *et al.,* 1993) were used to amplify the variable regions V3 of the 16S rRNA gene, giving PCR products of about 200 bp. To the forward primers, a GC clamp was added according to Muyzer et al. (1993). Each mixture (final volume, 25 µL) contained 1 µL of template DNA (16S rDNA amplification product), each primer at a concentration of 0.2 µM, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM $MgCl_2$, 2.5 µL of 10X PCR buffer (Invitrogen) and 2.5 U of Taq polymerase (Invitrogen). Template DNA was denatured for 5 min at 94°C. A "touchdown" PCR was performed as previously described (Aponte et al., 2009) to increase the specificity of amplification and to reduce the formation of spurious by-products. All amplifications were performed in a programmable heating incubator (Techno, Progene, Italy). Aliquots (5 µL) of PCR products were routinely checked on 2% agarose gels.

3.2.6.3 Denaturing Gradient Gel Electrophoresis (DGGE) analysis

PCR products were analysed by DGGE using a Bio-Rad D-code apparatus and the procedure first described by Muyzer et al. (1993). Samples were applied to an 8% (wt vol^{-1}) polyacrylamide gels in 1X TAE buffer. Parallel electrophoresis experiments were performed at 60°C by using gels containing 30-60% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40%, wt vol^{-1}, formamide) increasing in the direction of the electrophoresis. The gels were run for 10 min at 50 V and 4 h at 200 V, stained with ethidium bromide for 5 min and rinsed for 20 min in distilled water. Bands were visualized using a UV transilluminator (Gel Doc EQ, Bio-Rad).

3.2.6.4 Sequencing of DGGE bands

DGGE bands to be sequenced were purified in water according to Aponte et al. (2008). One µL of the eluted DNA of each DGGE band was re-amplified by using the above-described primers and conditions. PCR products which gave a single band co-migrating with the original band were then purified by QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions, and sequenced by dideoxy chain termination method (Sanger et al., 1977) by using reverse primer V3r described by Muyzer et al. (1993) by Gene Chron (Roma, Italy). Research for DNA similarity was performed with the National Centre of Biotechnology Information GenBank (Altschul et al., 1997).

3.2.6.5 LAB isolation, identification and genotypic characterization

A total of 52 of presumptive LAB colonies were randomly picked from MRS agar plates seeded with the highest sample dilutions. Each colony was purified by repeated streaking on the same medium. To extract DNA, LAB cultures were transferred to MRS broth, and incubated at 30°C for 48 hours. 2mL of broth cultures were used for the extraction by performing the same protocol adopted for extraction by cell bulks (Aponte et al., 2012). Prior to identification, strains were analyzed by RAPD-PCR (Random Amplified Polymorphic DNA) with M13 (5'-GAG GGT GGC GGT TCT-30) primer (Huey & Hall, 1989). Strains characterized by different DNA fingerprintings, were identified by 16S rDNA sequencing according to procedure described by Aponte et al. (2008) and using the forward primers (FD1) designed by Weinsburg et al. (199). DNA similarity was determined by BLAST search tool within the National Centre of Biotechnology Information, GenBank (Altschul et al., 1997).

3.2.7 Statistical analysis

All data obtained were statistically analysed by one-way ANOVA using SPSS version 17.0 (SPSSInc., Chicago, IL, USA). Analysis of variance was carried on in order to evaluate the effect of curing processes on microbial counts and pH. Significant differences between the treatment means were compared by means of Duncan's multiple comparison test at the 95% confidence level ($p \leq 0.05$). After 96 h of fermentation, statistical differences between two treatments (A and B) were evaluated by using a t- Student test where $p < 0.05$ was considered as statistical significant.

3.3 Results and discussion

3.3.1 Moulds identification

Moulds were randomly isolated from DRBC agar plates seeded with water curing samples and dilutions (10^{-1}) of chestnuts in sterile saline solution. D1/D2 and ITS sequencing allowed to unequivocally assigning just one strain (9M) to the species *Penicillium* (*Pn.*) *spinulosum* (similarity 100%). Four strains (1M, 3M, 4M and 5M) could be classified as *Pn. glabrum/Pn. purpurescens* (similarity 100%), four (2M, 7M, 8M and 10M) as *Pn. spinulosum/Pn. glabrum/Pn. crustosum* (similarity 99% at least), two (6M and C2) as *Pn. commune/Pn. crustosum* (similarity 98% at least), and one (C1) as *Pn. italicum/Pn. expansum* (similarity 100%). The high incidence of moulds referable to the genus *Pennicillium* spp. is in agreement with avaible references (Overy et al., 2003; Jermini et al., 2006; Migliorini et al., 2010).

3.3.2 Antifungal activity evaluation

According to evidences obtained by the "spot-on-lawn assay", antifungal activity appeared more widespread if strains were grown in anaerobic conditions. Within 44 analysed LAB, 27 strains were chosen since able to inhibit at least eight moulds out of twelve (Data not shown). The selected 27 strains were further evaluated for antifungal activity in two more stringent conditions: MRS with glucose reduced to 0.2% and MRS without sodium acetate. It was observed that several LAB strains producing large zones of inhibition on MRS agar plates, which means in the presence of 61 mmol L^{-1} sodium acetate, were unable to produce inhibition zones on MRS without this component and on MRS agar with reduced glucose content (Data not shown). On the basis of the recorded positive responses in both testes, 11 strains were further assayed by "mould agar spot assay" after growth in the same two media previously employed, namely MRS with less glucose (0.2%) or without sodium acetate (Table 4). According to data, a complete inhibition of moulds was never obtained, but a significant reduction of the mould diameter was observed in several cases (Negative values in Table 4). On the other hand, a more vigorous mould development was sometimes recorded in media containing filter-sterilized culture supernatants (Table 4), probably due to the enrichment in compounds of bacterial origin that may be metabolized by fungi. As general consideration, sodium acetate, a basic component of commercial MRS medium, proved to be able to exert a strong antifungal activity, as already reported (Stiles et al., 2002). Moreover, the degree of fungal inhibition could not be simply related to the acids production by LAB, thus confirming the highly complex nature of the antifungal activity (Dalié et al., 2012). Five strains, namely three *Lb. pentosus* isolated from table olives (OM13, OM24 and OM64) and two *Lb. paracasei* subsp. *tolerans* (1A and 1B) coming from water kefir grains andcommercial probiotic tablet, respectively, were selected for further tests.

3.3.3 Evaluation of strains ability to ferment in water curing environment

The ability of five selected strains to survive and develop in water curing environment was evaluated by microbial counting and pH measuring after 48 h. According to data, the best performances were exhibited by strain *Lb. pentosus* OM13, able to increase of about one Log (CFU mL^{-1}) the initial population level (from 5.98±0.41 to 7.32±0.54 Log CFU mL^{-1}) and to drop pH under 5.00 (from 6.06±0.71 to 4.90±0.13) in 48 h (Data not shown).

Table 4 Variation in moulds growth expressed as difference between the colonies diameter recorded on ME added of LAB culture supernatants and on ME agar with un-inoculated MRS broth. MRS adjusted to pH 4.00 by HCl or L-lactate addition was used as control.

LAB strain																			
			pH[a]	Mould strain								pH	Mould strain						
Species	Id.code			3M	5M	7M	8M	9M	10M	C1	C2		3M	5M	7M	8M	9M	10M	C1	C2
Lb. pentosus	OM13	MRS with 0.2% glucose	5.21	-2	0	1	3	4	1	3	6	5.16	-3	-2	-3	-4	-3	2	-8	-5
Lb. pentosus	OM24		5.23	-1	0	2	3	5	0	2	4	5.24	1	-5	-4	-3	-4	3	-6	-6
Lb. pentosus	OM50		5.17	0	1	3	0	4	-1	4	5	5.24	-2	-2	-2	-3	-1	-1	2	-7
Lb. coryniformis	OM60		5.24	1	3	3	4	3	2	3	5	5.52	3	-2	0	-5	-4	-1	-4	-6
Lb. pentosus	OM64		5.23	0	-2	-3	-3	5	-1	2	4	5.28	2	-5	-3	-6	-2	-2	-7	-7
Lb. casei	Lb491		5.23	-1	-1	2	0	5	1	4	5	5.24	-4	-4	1	-5	-3	-1	2	-5
Lb. Rhamnosus	RBM526		5.27	1	0	4	5	4	2	4	5	5.23	-3	-2	0	-5	-1	0	-3	-6
Lb. paracasei subsp. *tolerans*	1A		5.26	1	0	3	1	4	1	3	4	4.99	1	-5	-5	-4	-4	-3	-8	-6
Enterococcus durans	4AP		5.30	-2	-2	-1	4	5	1	3	5	5.90	-2	-3	0	-1	3	4	-5	-8
Lb. paracasei subsp. *tolerans*	1B		5.25	-1	2	4	3	3	2	4	3	5.19	-3	-2	-6	-4	0	-4	2	-5
Lb. Reuteri	L.R.		5.58	-2	2	0	2	5	1	2	3	6.26	3	-1	-3	0	-3	1	-7	-5
MRS + Lactic acid			4.00	-3	-1	-1	1	2	-4	-4	-2	4.00	-4	-5	-3	-2	-5	-1	-8	-9
MRS + HCl			4.00	-2	-1	0	0	3	-1	-2	-1	4.00	-2	-4	-3	-2	-1	-1	-6	-6

MRS without sodium acetate

[a]pH of filter-sterilized culture supernatants and control broths.

3.3.4 Curing processes on a laboratory scale

Three trials of water curing processing were performed on small batches (5 Kg): curing with adjunct of strain *Lb. pentosus* OM13 (A), conventional water curing with tap water (B), curing with water recycling (C). Water curing was considered concluded, when chestnuts were soaked up to the core, pH dropped around 5.00, and bubbles appeared. The curing with water recycling lasted just three days, while almost one week was required for the classic water curing (B) and for the process with the inoculation of *Lb. pentosus* as a starter (A). Results of pH, total aerobic microflora, *Enterobacteriaceae*, LAB, yeasts and mould are reported in Table 5. As expected, at the beginning of the curing (t = 0 h), microbial populations levels were higher in trial C with water recovery and, of course, pH presented the lowest values ($p< 0.05$). In general terms, LAB counts reflected the optimal adaptation of the starter strain to the environment: at the end of the monitoring, LAB loads were still higher than 6 Log CFU mL^{-1} in trial A. Yeasts and moulds stayed almost stable in trial C (5.3±0.92 to 4.83 ± 0.77 Log CFU mL^{-1}), while in the other two treatments (A and B) did not show significant differences ($p < 0.05$): after an initial increase, loads dropped to values around 4 Log CFU ml^{-1} (4.63±0.16 and 4.03±0.02 in A and B, respectively) (Table 5). In the trial C, *Enterobacteriaceae* stayed stable around 4.50 Log CFU ml^{-1} ($p< 0.05$).

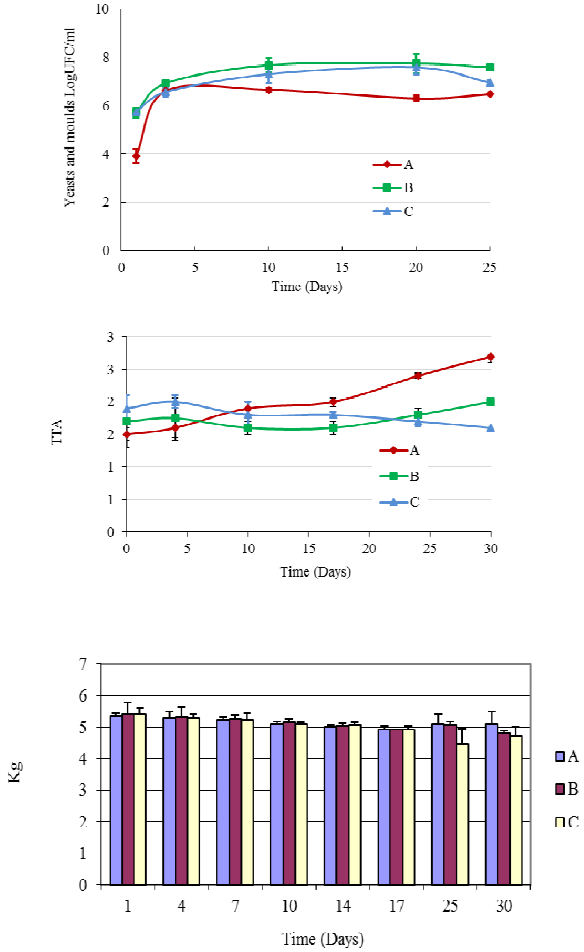
Table 5 Effect of water curing treatments (A, B, C) on pH and on microbial counts (Log CFU mL^{-1}) of chestnuts, cultivar (cv) "Castagna di Montella", during process (0-144 h). Experiments performed on a laboratory scale.

Media and incubation conditions	Trial[a]	Hours						
		0	24	30	48	72	96	144
pH	A	$^{z}7.20_{b}$±0.16	$^{y}6.04_{b}$±0.18	$^{x}5.75_{b}$±0.32	$^{x}5.58_{b}$±0.60	$^{x}5.57_{b}$±0.09	^{x,w}5.43±0.08*	^{w}5.21**±0.04*
	B	$^{z}7.15_{b}$±0.24	$^{y}6.10_{b}$±0.03	$^{y}6.00_{b}$±0.10	$^{y}5.91_{b}$±0.01	$^{y}5.81_{b}$±0.20	^{y}6.05±0.05*	^{x}5.48±0.03*
	C	$^{z}4.75_{a}$±0.02	$^{z}4.70_{a}$±0.12	$^{z}4.72_{a}$±0.08	$^{z}4.69_{a}$±0.05	$^{z}4.65_{a}$±0.07		
PCA (30°C; 48h)	A	$^{w}5.26_{a}$±0.12	$^{x}6.06_{a}$±0.71	$^{y}7.54_{b}$±0.06	$^{y}7.44_{a,b}$±0.12	$^{y}7.41_{b}$±0.04	^{y}7.38±0.18	^{z}8.02±0.04*
	B	$^{w}5.14_{a}$±0.36	$^{x}5.98_{a}$±0.10	$^{x}6.08_{a}$±0.08	$^{y}7.11_{a}$±0.16	$^{y}7.18_{a}$±0.02	^{y}7.23±0.10	^{z}7.57±0.03*
	C	$^{z}7.20_{b}$±0.16	$^{z}7.38_{b}$±0.42	$^{z}7.38_{b}$±0.22	$^{z}7.52_{b}$±0.21	$^{z}7.51_{b}$±0.06		
MRS (30°C; 48 h)	A	$^{v}2.23_{a}$±0.16	$^{w}2.55_{a}$±0.14	$^{z}7.56_{c}$±0.08	$^{y}6.66_{c}$±0.04	$^{x}6.46_{c}$±0.16	^{x,y}6.47±0.01*	^{x}6.13±0.06*
	B	$^{x}2.15_{a}$±0.14	$^{x,y}2.64_{a}$±0.48	$^{x,y}2.70_{a}$±0.11	$^{x,y}3.04_{a}$±0.56	$^{y,z}3.20_{a}$±0.06	^{y,z}3.26±0.30*	^{z}3.43±0.07*
	C	$^{z}5.02_{b}$±0.13	$^{z}5.13_{b}$±0.18	$^{z}5.13_{b}$±0.36	$^{z}5.15_{b}$±0.76	$^{z}4.57_{b}$±0.16		
DRBC (28°C; 5 days)	A	$^{w}3.95_{a}$±0.21	$^{w}3.98_{a}$±0.34	$^{x}4.54_{b}$±0.09	$^{y}5.03_{a}$±0.23	$^{z}6.27_{b}$±0.30	^{y}5.22±0.16*	^{x}4.63±0.16*
	B	$^{x}3.70_{a}$±0.33	$^{x}3.89_{a}$±0.76	$^{x}3.90_{a}$±0.08	$^{y}4.78_{a}$±0.44	$^{z}5.66_{a,b}$±0.11	^{z}5.96±0.01*	^{x}4.03±0.02*
	C	$^{z}5.30_{b}$±0.92	$^{z}5.03_{b}$±0.07	$^{z}5.18_{c}$±0.45	$^{z}5.19_{a}$±0.13	$^{z}4.83_{a}$±0.77		
VRBGA (37°C; 24 h)	A	$^{x}3.57_{a}$±0.35	$^{x}4.48_{a}$±0.56	$^{x}4.18_{a}$±0.86	$^{z}5.78_{a}$±0.26	$^{z}5.84_{b}$±0.93	^{z}6.04±0.02	^{y,z}5.32±0.32
	B	$^{y}3.78_{a}$±0.97	$^{y}4.00_{a}$±0.89	$^{y}4.00_{a}$±0.74	$^{z}5.95_{a}$±0.54	$^{z}5.71_{a,b}$±0.08	^{z}6.01±0.25	^{z}5.21±0.37
	C	$^{z}4.26_{a}$±0.76	$^{z}4.25_{a}$±0.66	$^{z}4.54_{a}$±0.81	$^{z}5.19_{a}$±0.49	$^{z}4.68_{a}$±0.08		

[a]A: Water curing with adjunct of strain *Lb. pentosus* OM13; B: conventional water curing with tap water; C: water curing with water recycling. Within each trial, means with different column subscripts (a-c) or row superscripts (v-z) are significantly different ($p< 0.05$).
*$p< 0.05$: significant differences (t-Student) are shown between A and B trial means.

After curing, chestnuts were monitored for the first 30 days of drying. Yeasts and moulds tracking revealed a rapid increase (1-3 Log CFU g^{-1}) during the first days of drying in all cases (Figure 2); at the end of the process, chestnuts coming from curing with inoculation of the starter culture presented a level of yeasts and moulds contamination more than one Log lower, if compared to classical curing, and a titratable acidity value considerably higher, if compared with chestnuts belonging to the other two batches (Figure 2). Moreover, after one month weight loss was about 10% and 13% for chestnuts coming from trial B and C, respectively, while was around 5% for chestnuts cured in presence of strains OM13 (Figure 2). As general consideration, colour and lustre of the epicarp of the chestnut produced in the three trials appeared different, even at glance. In detail, nuts coming from the starter added curing exhibited an unusual lustre, thus appearing as untreated. In fact, it is widely accepted that water curing process makes the treated fruit lose the natural lustre (Jermini et al., 2006).

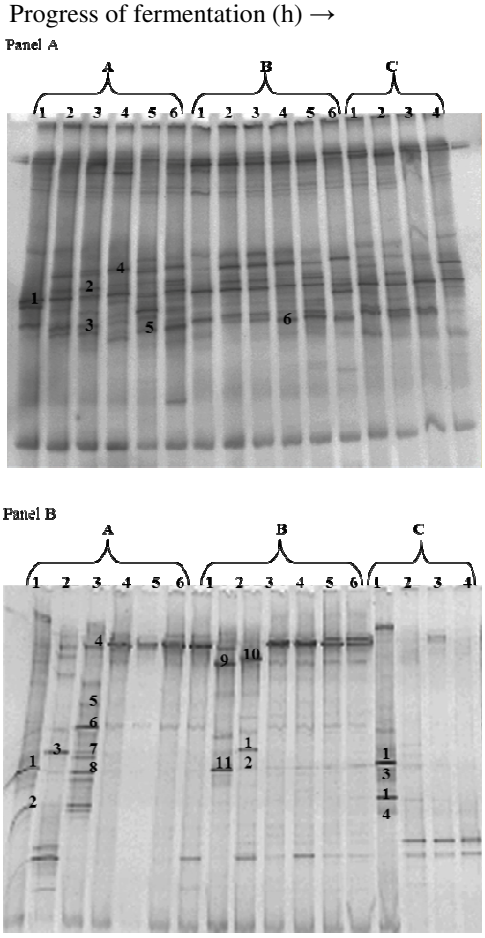
Figure2 Evolution of yeasts and moulds on DRBC agar, of total titratable acidity (TTA) and of weight loss during drying of chestnuts deriving from the curing process performed on pilot scale. Each point in the figure is a mean value ± standard deviation. A: Curing starter added; B: Conventional curing with tap water; C: Curing with water recycling.



Total microflora on PCA (Figure 3, panel A) and LAB on MRS plus cycloheximide (Figure 3, panel B) were evaluated by means of a PCR-DGGE-based approach. With reference to LAB evolution, when the inoculum with *Lactobacillus* strain OM13 was performed, an increase in the microbial biodiversity of the trial A (30 h) was detected (Figure 3, panel B). Afterward, samples collected from the trial with adjunct culture, always showed extremely poor patterns, while trial B and above all trial C, exhibited DGGE profiles with two or more major bands up to the end of the process (Figure 3, panel B). Identification by blast comparison on NCBI gene bank of fourteen DGGE major bands from MRS cell bulks, allowed the detection of seven taxons (Figure 3). One band, identified at group level as *Lb. pentosus* (100% of similarity with the following species: *Lb. fabifermentans, Lb. plantarum, Lb. plantarum* subsp. *argentoratensis, Lb. paraplantarum* and *Lb. pentosus*), was retrieved, as expected, in all samples of the trial A collected after the inoculum (24, 30, 48, 72 and 144 h). Indeed, bands at the same height were even visible in trial B by the day three. This statement comes as no surprise, since members of this *Lactobacillus* group are known to be able to survive in several hostile environments and, in detail, the employed *Lb. pentosus* strain (OM13) is presently used to led lactic fermentation in Spanish-style green olive production at industrial level (Aponte et al., 2012). Apart from lactobacilli, the sole further LAB detected in water curing environment was *Lactococcus (Lc.) lactis* subsp. *hordniae*, a species quite common on plants or within the digestive tract of cows. As any other product strictly related to the soil, water curing appear to be a suitable environment for spore-forming bacteria (Figure 3). In detail, four and seven bands from PCA and MRS cell bulks, respectively, could be related genus *Bacillus*, two bands from MRS cell bulks could be reported to the genus *Rummelliibacillus* spp and four from PCA to the genus *Paenibacillus* spp. In detail,five bands from MRS and two from PCA cell bulks appeared related (99% of similarity at least) with *Bacillus* (*B.*) *mycoides, B. anthracis* and *B. thuringiensis,* three species referable to the so-called *Bacillus cereus* group. *B. cereus* is naturally present in some soils, so its occasional presence on fruits and vegetables should not be unexpected (Mongkolthanaruk, 2012). Two bands from MRS cell bulks appeared related

(99% of similarity at least) with *B. amyloliquefaciens, B. vallismortis* and *B. atrophaeus,* all belonging to the so-called *Bacillus subtilis* group comprising closely related species plant-associated (Mongkolthanaruk, 2012).
Members of the genus *Rummeliibacillus* spp. have been recently found in soil (Her & Kim, 2013), while *Paenibacillus* spp. have been detected in soil, water, rhizosphere and a variety of food (Postollec et al., 2012). Almost all samples from MRS cell bulks presented a band at the same height, which resulted referable to *Acinetobacter* spp. (Figure 3). Species of this genus are ubiquitous within the environment and are found in soil, water, and on the dry surfaces of diverse devices (Choi *et al.,* 2012). The ability to persist in several environments makes *Acinetobacter* species one of the most common pathogens in hospitals (Choi et al., 2012).

Figure3 DGGE of V3 amplicons. DNA extracted from cell bulks collected from PCA (Panel A) and MRS (Panel B) agar plates. Experiments performed on a laboratory scale (A: Curing with inoculation of the starter culture, B: Conventional curing; C: Curing with water recycling).



Panel A Panel B

Band n.	Closest relative	% sequence similarity	Band n.	Closet relative	% sequence similarity
1,5	*Bacillus cereus* group	99-100	1,5,7,11,13	*Bacillus cereus* group	99-100
2	*Enterobacter ludwigii*	99	2,14	*Bacillus subtilis* group	99-100
3	*Bacillus idriensis*	99	3,12	*Lc. lactis* spp *hordniae*	100
4	*Paenibacillus lactis*	100	4	*Lb. pentosus*	100
6	*Bacillus safensis*	99	6	*Acinetobacter calcolaceticus*	97
			8	*Delftia tsuruhatensis*	100
			9,10	*Rummelliibacillus stabekisii*	100

3.3.5 Curing processes on a farm scale

The same set of trials was repeated on a farm scale on batches of chestnuts of about 200 Kg each. In this case, process lasted five days in all cases. Differences in the nuts colour and lustre were noted in this case as well. Data about pH and microbial evolution are reported in Table 6. Results for pH reflected, at the beginning of the curing, the same conditions registered in experiments on a pilot scale, but values recorded at the end were significantly lower ($p< 0.05$) in trial A and B, even if the process lasted two days less. On the other hand, yeasts and moulds, at the end of the process resulted lower in the curing with inoculation of the starter culture (A) (Table 6).

Table 6 Effect of water curing treatments (A, B, C) on pH and on microbial counts (Log CFU mL^{-1}) of chestnuts, cultivar (cv) "Castagna di Montella", during process (0-96 h). Experiments performed on a farm scale.

Media and incubation conditions	Trial[a]	Hours				
		0	24	48	72	96
pH	A	$^{z}7.02_{b}\pm0.29$	$^{y}5.66_{a}\pm0.32$	$^{x}4.80_{a}\pm0.66$	$^{x}4.85_{a}\pm0.31$	$^{x}4.85_{a}\pm0.24$
	B	$^{z}7.03_{b}\pm0.56$	$^{z}6.70_{b}\pm0.81$	$^{z,y}6.19_{b}\pm0.54$	$^{y}5.45_{a}\pm0.82$	$^{y}5.37_{a}\pm0.91$
	C	$^{z}5.98_{a}\pm0.31$	$^{z,y}5.51_{a}\pm0.09$	$^{y,x}5.10_{a}\pm0.25$	$^{y,x}4.95_{a}\pm0.13$	$^{x}4.74_{a}\pm0.65$
PCA (30°C; 48 h)	A	$^{y}5.70_{b}\pm0.84$	$^{z}6.78_{b}\pm0.37$	$^{z}7.15_{b}\pm0.34$	$^{z}7.45_{b}\pm0.31$	$^{z}7.08_{a}\pm0.48$
	B	$^{x}3.72_{a}\pm0.76$	$^{y}5.13_{a}\pm0.47$	$^{y,z}5.90_{a}\pm0.29$	$^{z}6.29_{a}\pm0.14$	$^{z}6.62_{a}\pm0.67$
	C	$^{x}5.78_{b}\pm0.24$	$^{x,y}6.48_{b}\pm0.26$	$^{y,z}6.65_{b}\pm0.44$	$^{z}7.36_{b}\pm0.25$	$^{z}7.29_{a}\pm0.79$
MRS (30°C; 48 h)	A	$^{w}2.28_{a}\pm0.04$	$^{z}8.23_{c}\pm0.34$	$^{y}7.06_{c}\pm0.33$	$^{x}5.92_{b}\pm0.54$	$^{x}5.69_{b}\pm0.82$
	B	$^{x}2.55_{a}\pm0.28$	$^{x}2.40_{a}\pm0.29$	$^{x}2.79_{a}\pm0.34$	$^{y}4.24_{a}\pm0.25$	$^{z}5.07_{a,b}\pm0.29$
	C	$^{x}3.45_{b}\pm0.28$	$^{x}3.47_{b}\pm0.27$	$^{x,y}3.80_{b}\pm0.31$	$^{y,z}4.11_{a}\pm0.24$	$^{z}4.58_{a}\pm0.24$
DRBC (28°C; 3-4 days)	A	$^{x}3.35_{b}\pm0.13$	$^{z}4.60_{b}\pm0.29$	$^{z}4.60_{b}\pm0.31$	$^{z}4.78_{a,b}\pm0.31$	$^{y}3.85_{a}\pm0.22$
	B	$^{x}2.67_{a}\pm0.38$	$^{x}2.63_{a}\pm0.51$	$^{y}3.70_{a}\pm0.30$	$^{z}5.00_{b}\pm0.26$	$^{z}5.30_{b}\pm0.30$
	C	$^{z}4.70_{c}\pm0.29$	$^{z}4.70_{b}\pm0.29$	$^{z}4.34_{a,b}\pm0.37$	$^{z}4.26_{a}\pm0.34$	$^{z}4.27_{a}\pm0.26$
VRBGA (37°C; 24 h)	A	$^{x}3.44_{b}\pm0.31$	$^{x,y}3.70_{b}\pm0.08$	$^{y}4.00_{b}\pm0.25$	$^{z}5.47_{b}\pm0.36$	$^{z}5.52_{b}\pm0.32$
	B	$^{x}2.11_{a}\pm0.14$	$^{x,y}2.35_{a}\pm0.76$	$^{y}3.00_{a}\pm0.28$	$^{z}4.14_{a}\pm0.26$	$^{z}4.30_{a}\pm0.38$
	C	$^{x}3.45_{b}\pm0.04$	$^{x,y}3.72_{b}\pm0.58$	$^{x,y}3.90_{b}\pm0.36$	$^{y}5.12_{b}\pm0.29$	$^{z}4.28_{a}\pm0.28$

[a]A: Water curing with adjunct of strain *Lb. pentosus* OM13; B: conventional water curing with tap water; C: water curing with water recycling.

Within each trial means with different column subscripts (a-c) or row superscripts (w-z) are significantly different ($p< 0.05$).

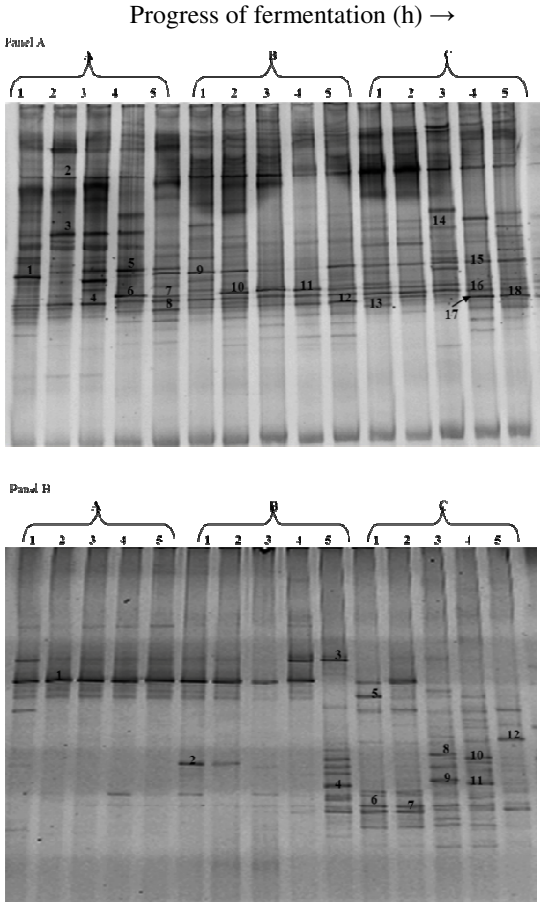
Even in this case, total microflora on PCA (Figure4, panel A) and LAB on MRS (Figure 4, panel B) were daily monitored by means of PCR-DGGE performed as previously described on cells collected on filters. Analysis of Figure 4 allows some interesting considerations. First of all, DGGE analysis of the same set of experiments on an industrial scale showed that, when curing waters is recycled, a complex microbial community colonizes the ecological niche.

In sample C, microflora proved to be dominated by *Enterobacteriaceae*, with particular regard to the genera *Serratia,Kluyvera, Raoultella, Budvicia* and *Enterobacter* spp. (Figure 4, panel A and B). *Raoultella terrigena* has been mainly reported as an aquatic and soil organism (Murray et al., 2005), while freshwater sources, including wells, water pipes, swimming pools, brooks and rivers are a selective environment for *Budvicia aquatica* (Schubert & Groeger-Sohn, 1998), so the presence of both species does not surprise in this environment. On the other hand, isolates of *Kluyvera*, even if found free-living in environmental settings, may possess resistance genes with high identity to those of pathogens (Forsberg et al., 2012). The second consideration regards the presence of one major group of LAB species in this ecosystem, the same employed as adjunct culture: the *Lb. pentosus* group (Figure 4, panel A and B).

Indeed, in the trial A, the inoculated microorganism appeared clearly dominant and the minor bands, visible at time zero, completely disappeared during the process. In the traditional curing process (B), even if present, lactobacilli coexisted with other LAB species, such as *Lc. lactis* subsp. *hordniae*, and *Streptococcus* (*St.*) *gallinaceus*, as well as with members of the *Enterobacteriaceae* family (Figure 4b). Spore-forming bacteria were less represented, if compared to DGGE analyses performed on pilot scale trials. The unique species recorded in MRS cell bulks was *B. flexus* in samples from trial C (Figure 4, panel B). On the other hand, the PCR-DGGE profiles of V3 rDNA amplicons obtained from DNAs extracted by cell bulks collected from PCA plates appeared quite complex (Figure 4, panel A). Moreover, the microflora retrieved in the three treatments was, with very few exceptions, largely dissimilar (Figure 4, panel A). Samples B and A, at time 0, appeared almost identical, but after the inoculum of the adjunct culture, at the day 2, the profiles of the two trials diverged. The completely independent ecosystem, referable to experiments with water recycling (C), was confirmed, as well as the prevalence of spore-forming bacteria: five bands out of 18 could be reported to the genus *Bacillus* spp., and four to the genus *Paenibacillus* spp.

(Figure 4, panel A). In other words, 50% of the detected species could be referred to the bacilli. Beyond *B. mycoides, B. idrensis* and *B. thuringiensis*, reported in Figure 4 as *B. cereus* group, a new species was retrieved: two bands exhibited a percent of similarity of 99% *B. pumilus*. This species, commonly found in soil and closely related to *Bacillus safensis* (Satomi et al., 2006), was already detected in the water curing process conducted on a pilot scale. Quite widespread on DGGE profiles, independently from the considered trial, was again *Enterobacter ludwigi*, an opportunistic pathogen belonging to *Enterobacteriaceae*. Another detected species of clinical relevance was *Salmonella enterica* subsp. *diarizonae.*

Figure 4 DGGE of V3 amplicons. DNA extracted from cell bulks collected from PCA (Panel A) and MRS (Panel B) agar plates. Experiments performed on a farm scale. (A: Curing with inoculation of the starter culture; B: Conventional curing; C: Curing with water recycling.

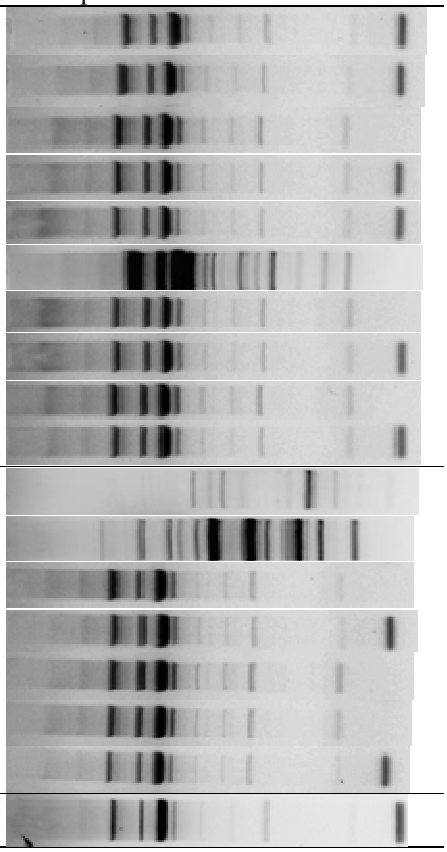


Panel A			Panel B		
Band n.	Closest relative	% sequence similarity	Band n.	Closet relative	% sequence similarity
1	*Aquaspirillum serpens*	97	1	*Lb. pentosus* group	100
2	*Lb. pentosus* group	100	2	*Lc. lactis* spp *hordniae*	99
3	*Lb. paracollinoides*	99	3	*Streptococcus bovis* group	100
4,12	*Stenotrophomonas maltophilia*	99	4	*Enterobacter asburiae*	99
5	*Paenibacillus lactis*	100	5	*Weissella soli*	99
6,10,11	*Bacillus safensis* group	99	6	*Serratia liquefaciens* group	99
8,13,17	*S. enterica/Ent. ludwigii*	100	7	*Budvicia acquatica*	98
14	*Psychromonas artica*	99	8,9	*Bacillus flexus*	99-100
9,15	*Bacillus cereus* group	100	10	*Kluyvera cryocrescens*	100
7,16,18	*Paenibacillus tundrae*	100	11	*Raoultella planticola* group	99
			12	*Pediococcus pentosaceus*	100

3.3.6 Evaluation of microbial dynamics by means of conventional isolation procedures

Fifty-two presumptive LAB were isolated from MRS agar plates seeded with the highest dilutions in order to get information about the dominant lactic microflora. In spite of the presence of cycloheximide in the medium, 35 cultures proved to be yeasts by microscope analysis. As consequence no LAB was retrieved by trial C. This limit of the cycloheximide in preventing the yeast growth has already been reported in other environments largely colonized by yeast, such as table olives (Aponte et al., 2012). Fifteen strains appeared as prokaryotic cells at the microscope and were submitted to strain typing (Table 7). RAPD analysis revealed a very low polymorphism among strains, as already recorded by other authors (Aponte et al., 2012; Panagou et al., 2008). Thirteen strains out of 15 showed a very similar pattern: in detail eight strains (A9, A11, A13, A15, A21, A23, B13, B21) exhibited a DNA fingerprint absolutely identical to the one of the adjunct culture, *Lb. pentosus* OM13 (Table 7). Six strains (A12, A20, A22, B12, B19, B20) presented a slightly different profile, if compared to strain OM13, since the band of the lowest molecular weight was missing. Three strains (A18, B5 and B6) showed completely different profile. Five strains representative of each detected RAPD profile were selected for 16S rDNA sequencing. Strain B12 characterized by a pattern slightly different from OM13 and strain A9, whose DNA fingerprinting perfectly matched the one of OM13, proved to be *Lb. pentosus*(99% of similarity). Strains, characterized by unique patterns (A18, B5 and B6) resulted to be *Lb. paracollinoides*, *Pseudomonas lini* and*Psychromonas arctica*, respectively (99% of similarity at least). *P. lini*, a new species isolated from soil (Delorme et al., 2002) is ubiquitous, while *Psychromonas artica*was even detected, by DGGE-based techniques, in PCA bulks. Starter monitoring by RAPD-PCR provided valuable evidence of the adaptation of the *Lb. pentosus* strain used as starter, even though its RAPD profile was recovered in two strains (B13 and B21) isolated during traditional water curing process (B). These results confirmed the prevalence of LAB, referable to the so-called *Lb. pentosus* group, in the water curing ecosystem.

Table 7 LAB cultures, source of isolation and RAPD profile.

Code	Trial	Day of curing	Population level (CFU mL^{-1})	RAPD profiles
A9[a]	A	1	10^2	
A11		2	10^6	
A12		2	10^6	
A13		3	10^5	
A15		3	10^6	
A18[a]		4	10^4	
A20		4	10^5	
A21		4	10^5	
A22		4	10^6	
A23		4	10^6	
B5[a]	B	1	10^2	
B6[a]		1	10^2	
B12[a]		3	10^3	
B13		3	10^3	
B19		3	10^3	
B20		3	10^3	
B21		3	10^3	
OM13	Adjunct culture			

[a]Strains submitted to 16S rDNA sequencing

3.4 Conclusions

Chestnuts deteriorate very easily: a rapid loss of moisture and/or attacks of nut weevil and mould parasites may compromise both sensory and nutritional properties. The traditional water curing may have inhibiting effects on fruit degradation. Hydration

of fruit during water curing produces a decreased moisture loss during storage and exerts an inhibiting effect on fruit infecting fungi. This work represents to authors' knowledge, the first attempt to produce a phenomenological hypothesis based on a microbiological point of view.

According to obtained evidences, in spite of the stringent conditions characterizing the ecological niche, a consistent microflora is able to colonize curing waters. As a matter of fact, microbial loads increase even in the classic *curatura* process and this acquisition appear to be divergent with the decreasing microbial counts reported by Migliorini et al. (2010). In addition, curing waters appear as a suitable environment for three main group of bacteria: spore-forming bacteria (*Bacillus*, *Paenibacillus*, and *Rummelliibacillus* spp., three strictly correlated genera), *Enterobacteriaceae* and *Pseudomonaceae,* and, at less extent, LAB, all bacteria that may be put in relation with soil or with surface waters.

When for technological and ecological reasons, a waters recycling is attempted, a reduction in the length of the curing process may be achieved since a sort of backsloping procedure take place. Nevertheless, according to evidences obtained by DGGE-based approach, this length contraction appears dearly. The microflora selected in these conditions appeared strongly dominated by spore-forming bacteria and above *Enterobacteriaceae* with several entities known as opportunist pathogens. Of course, since chestnut are dried before commercialization, cooked before consumption (boiled or roasted) and ingested after the removal of the pericarp, the real risk does not regard consumers but operators in touch with the water curing vessels.

On the other hand the employment of an adjunct culture appears as a very promising opportunity. The starter monitoring by PCR-DGGE, as well as by RAPD-PCR, provided valuable evidence of the adaptation of strain used as starter. Even if no reduction in the length of the *curatura* process was achieved in these conditions, nor in the water curing on a laboratory scale, neither in the one performed at industrial level, it must be stressed that some interesting results emerged anyway. In particular, according to observations in both set of trials, waters treated with adjunct culture exhibited, in general terms, a minor microbial complexity and surprisingly, fruits did not lose luster after the process.

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3.6 Production of novel and functional foods by fermentation of chestnut-based matrices

Many traditional products that once used to be strictly connected to the needs and consumption behaviour of low-income people are now regarded as natural, healthy products (FAO, 1995). The general increase in demand for "natural" products is a driving force that also has a positive influence on the chestnut market. At the same time, most of the commercially available probiotic carriers are dairy-based products, such as yoghurt, fermented milk, ice cream and cheeses. One of the main setbacks of consuming dairy products by those with deficiency in the enzyme β-galactosidase is lactose intolerance, in which lactose cannot be digested and absorbed,leading to abdominal cramping, bloating, diarrhoea and nausea. For this reason an increasing interest is currently found for different food matrices which may offer a number of advantages (Prado et al., 2008).

In this study, two alternative industrial approaches were evaluated to attribute value added to overproduction of chestnuts: the development of chestnut-based purees fermented by selected LAB strains and the production of alcoholic beverages from distillation of fermented chestnut (López et al., 2004, 2006; Murado et al., 2008).

3.7 Materials and Methods

3.7.1 Microorganisms and culture conditions**.**

LAB strains used in this study were as follows: *Lactobacillus* (*Lb*.) *rhamnosus* VT1, RBM526, and RBT739 isolated from Parmigiano Reggiano cheese and kindly provided by DISTAAM (Campobasso, Italy), *Lb. casei* Lbc491 and Lbc496, isolated from Provolone del Monaco cheese (Aponte et al., 2008) and *Lb. rhamnosus* GG from ATCC (ATCC 53103). All isolates were stored at -25°C in liquid MRS (Oxoid) after addition of 20% sterile glycerol.

3.7.2 Chestnut purees preparation and fermentation

Chestnut purees (CP) were prepared as follows: broken dried chestnuts were boiled in tap water (1:3; wt vol^{-1}) for about one hour, drained, mixed in the ratio 1:2 (wt vol^{-1}) with the boiling-derived water and homogenized by ultra-turrax. After distribution in pot (50 mL), purees were sterilized by autoclave (121°C for 15 min, 1 Atm). Chestnut purees (CP) were inoculated (2%) with overnight cultures, of the six strains in MRS or in CP, and incubated at 30°C. Samples were taken at 0, 7 and 24 h for determination of pH, *Lactobacillus* spp. loads by plating in duplicate on MRS agar and reducing sugars content by HPLC. HPLC Analysis was performed on samples diluted in mobile phase 1:5 (vol vol^{-1}) and filtered by AcroDisc millipore (0.2 μm) by system (Gilson 307 Series HPLC system) fitted with column (MetCarb68Hcolumn 6.5x300 mm, Varian) in an oven thermostated at 65°C. Columns were eluted at 0.4 mL min^{-1} by a solution 1:9 (vol vol^{-1}) of H_2SO_4 in ultrapure water. A refractometer (RID 133, Gilson) was used as detector. Standards were used for quantification of different sugars and acids in the samples. Each experiment was performed in triplicate. After fermentation, purees were stored at 4°C. The vitality of lactobacilli along storage time was evaluated by counting on MRS agar plates at 1, 7, 15, 21 and 40 days of storage.

3.7.3 Preliminary organoleptic evaluation

A preliminary organoleptic analysis was performed on samples of 24 h fermented CPs after 6 h of cooling at 4°C. To define the sensory profile (UNI 10957, 2003), a descriptive panel of 15 judges (9 females and 6 males, aged between 24 and 30 years) was employed. The judges were trained in three preliminary sessions in order to produce a list of attributes useful to define the sensory profile of fermented chestnut purees. On the basis of the frequency of citation (> 60%), eight descriptors were selected to be inserted in the card: colour texture, flavour, off-flavour, taste, chestnut perception, sour and overall. Random samples were evaluated by assigning a score between 1 (absence of the sensation) and 10 (extremely intense).

3.7.4 *Lactobacillus* strains survival under conditions simulating the human gastrointestinal tract

The effect of low pH was examined by the method of Kimoto et al. (1999). Cells of *Lactobacillus* strains were harvested from MRS overnight cultures (5 mL) by centrifugation (1840 *g* for 10 min) and resuspended in 0.85% NaCl solution at pH 2.0, 2.5 and pH 3.0 adjusted with 1 N HCl, and then held at 37°C for 30 min. The number of surviving cells was determined by a plate count on MRS agar. Saline solution at pH 6.0 was used as control. The sensitivity of strains to bile was tested on MRS agar plates containing 0.15 and 0.3 of Oxgall (Sigma-Aldrich; St. Louis, MD, USA), and on unmodified MRS agar as control; cells were harvested by centrifugation of MRS broth overnight cultures and resuspended in saline at pH 6.0. A combined trial was arranged by keeping cell suspensions in saline at pH 2.5 for 30 min at 37°C, followed by plating on MRS agar added of 0.3% of Oxgall. In all trials counting plates were incubated at 30°C, for 24-48 h, and the number of colonies counted. Each experiment was performed in triplicate.

3.7.5 Antibiotic susceptibility

The antibiotic-susceptibility profile of strains was tested using the disk diffusion method on Mueller-Hinton agar, according to the NCCLS guidelines (2002). The antibiotics used and their concentrations were as follows: amoxicillin (25 μg), ampicillin (10 μg), aztreonam (30 μg), bacitracin (10 μg), carbenicillin (50, 100 μg), ceftazidime (30 μg), cefoperazone (15 μg), cefoxidin (30 μg), cephaloridine (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), clindamycin (2 μg), cloxacillin (1 μg), erythromycin (15 μg), fosfomycin (50 μg), furazolidine (100 μg), fusidic acid (10 μg), gentamicin (10 μg), kanamycin (30 μg),

imipenem (10 μg), lincomycin (2 μg), metronidazole (80 μg), mezlocillin (75 μg), netilmycin (30 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), novobiocin (30 μg), oleandomycin (15 μg), oxacillin (1 μg), oxytetracycline (30 μg), penicillin-G (10 μg), pipemidic acid (20 μg), piperacillin (100 μg), polymixin (300 mg), rifampicin (30 μg), tetracycline (30 μg), chlorotetracycline (30 μg), spiramycin (100 μg), streptomycin (10 μg), sulfamethoxazole (100 μg), tobramycin (10 μg), and vancomycin (30 μg).All antibiotics were provided by BioMérieux SA, (Marcy l'Etoile, France).

3.7.6 Starch, protein and lipid digesting capabilities

Extracellular proteolytic activity and utilization of starch were assessed by using Plate Count Agar with 50% Skim Milk (Fadda et al., 2004) and starch agar, respectively. For lipolytic activity two different protocols were used (Haba et al., 2000; Kouker & Jaeger, 1987). In all cases, overnight cultures in MRS (10 μL) were dropped on the modified agar surface and incubated at 30°C for 48 h. The diameters of the halo zone on the agar plate were then measured. The digesting capability of the tested strains was classified as positive when the diameters of clear zone were more than 1 mm. Each assay was performed in triplicate.

3.7.7 Features of technological interest

Strains were screened for citrate metabolism using Simmons Citrate Agar (Oxoid, Basingstoke, United Kingdom). Acetoin production was evaluated by using the Voges-Proskauer test on overnight cultures of the strains in MR-VP, MRS, MRS enriched with citrate (0.8%) or with skim milk (1%) as well as in CP. Screening for decarboxylase activity was performed as reported by Joosten and Northolt (1989) with ornithine, histidine, lysine and tyrosine (Sigma, Milan, Italy) as decarboxylase substrates. Exopolysaccharide production was tested in ST1 medium (Gancel et al., 1994) supplemented with filter-sterilized solutions of different carbohydrates (1% vol vol^{-1}) and ruthenium red (0.08 g L^{-1}). Glucose, lactose, sucrose, starch, skim milk powder and a mixture of equivalent concentrations of glucose, lactose and sucrose were added one by one.

3.7.8 Antibacterial activity

Strains were screened for antibacterial activity by agar-spot deferred test (Schillinger & Lücke 1989) with *Listeria innocua* 1770, *Pseudomonasfragi* 6P2, *Carnobacterium* spp. 9P, *Brochotrixthermospacta* 7R1, *Escherichiacoli* 32, *Hafniaalvei* 53M, *Serratiaprotom* 42M, *Staphylococcus* spp. 3S, and *Salmonellatyphimurium* as indicator strains. Each assay was performed in triplicate.

Sensitivity of the antimicrobial compounds to proteolytic enzymes was tested by adding, close to the microbial spot, a drop of a mixture of the following proteolytic enzymes: protease, pronase, pepsin, chimopapain, α-chimotripsin and trypsin (Sigma-Aldrich) at a final concentration of 10 mg mL^{-1}. Moreover, overnight bacterial cultures were harvested by centrifugation (4000 *g*, 10 min at room temperature), neutralized to pH 6.5 with 1 N of NaOH to rule out acid inhibition, filtered through a 0.22 μm-pore-size cellulose acetate filter (Sartorius, Goettingen, Germany) to obtain a cell-free supernatant and finally treated at 80°C for 5 minutes. Inhibitory activity from hydrogen peroxide was ruled out by the addition of catalase (300 IU mL^{-1}) (Sigma-Aldrich) using the well diffusion assay (Schillinger & Lücke 1989). Briefly, TSA soft agar medium containing the indicator strain, as reported above, was poured in plates. Thereafter wells (6 mm in diameter) were cut into the agar and 50 μL of the cell-free supernatant of the potential producer strains was placed into each well. For bacteriocin-like substances extraction, further four approaches were tried. Firstly, the acid extraction of cell-associated bacteriocins was attempted according to the method proposed by Jack and Tagg (1992) properly modified: briefly, cells grown under appropriate conditions were harvested by centrifugation at 6000 g for 20 min, washed twice in 5 mM phosphate buffer, pH 6.0-8.0, and resuspended to half of the original culture volume in HCl 50 mM. After 18 h at 4°C, the cells were removed by centrifugation and the bacteriocin-containing supernatant was assayed. A second extraction was carried out according to protocol proposed by Rodríguez et al. (2010) modified as follows: strains were cultured in CGB medium (Huot et al., 1996), then fermentation broth was acidified to pH 2.0 with 1 N HCl, heated at 100°C for 15 min and centrifuged (6000 *g* for 15 min). Surnatants were neutralized and assayed. As third approach, the protocol developed by Corsetti et al. (2004) for the detection of antimicrobial activity in sourdough, was modified as follow: cultures in broth were spread on TSA agar soft plates. After colony growth, agar was removed and resuspended in ratio 1:9 (wt vol^{-1}) in a 40% acetonitrile-0.1 % (vol vol^{-1}) trifluoroacetic acid solution, homogenized, centrifuged at 15.000 g for 10 min, freeze-dried and finally resuspended in 5.5 mL of a 40% (vol vol^{-1}) ethanol solution and then assayed. As last attempt, agar from well-grown TSA agar cultures was removed and treated by stomacker, one mL was centrifuged (10.000 rpm for 3 min.) and assayed while one mL was treated with isopropanol (70%), centrifuged (10.000 rpm for 3 min.) and assayed. In all cases antimicrobial activity was detected by the agar-spot deferred test and the well diffusion assay (Schillinger & Lücke 1989), as previously described.

Research for reuterin (3-hydroxypropionaldehyde) was performed according to Martin et al. (2005); results were confirmed by PCR detection of the gene encoding for a glycerol dehydratase subunit according to the protocol developed by Claisse and Lonvaud-Funel (2001). DNA from *Lb. reuteri* CECT 925T was used as positive control.

3.7.9 Strains genotyping by PFGE analysis

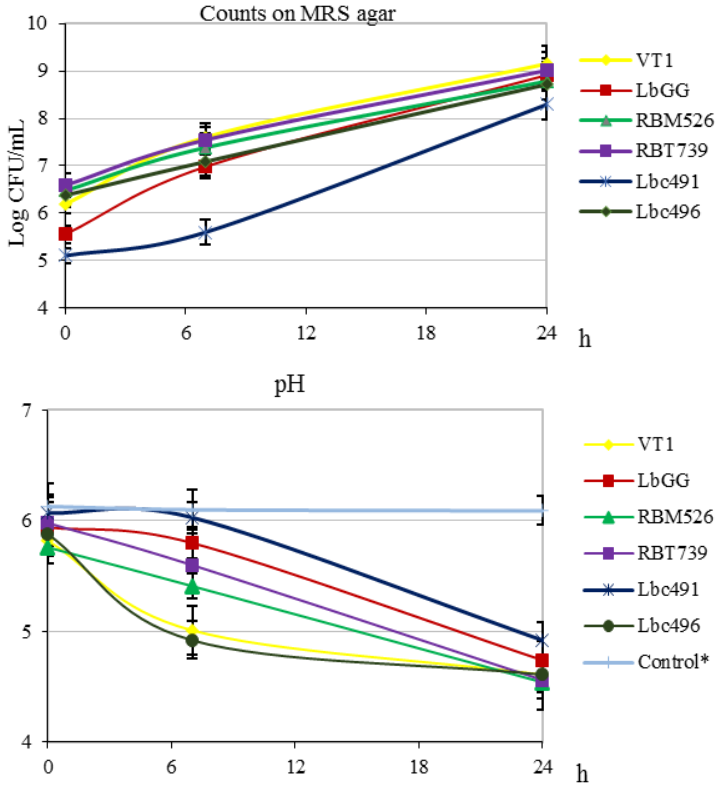
Preparation of agarose plugs containing chromosomal DNA for PFGE analysis was carried out by the method of Blaiotta et al. (2001). The inserts of intact DNA were digested in 200 µL of appropriate buffer supplemented with 40 U of *Sma* I (Promega) at 25°C for 18 h. Genomic DNA fragments were separated by PFGE at 9°C on agarose 1% wt vol^{-1} gels in a Clamped Homogeneous Field Electrophoresis apparatus (CHEF-DRII system; Bio-Rad, Hemel Hempstead, UK). Restriction fragments were resolved in a single run, with pulse times ramping from 1 to 10 s over 16 h at 6.0 V/ cm in 0.5X TBE (45 mmol L^{-1} Tris-borate, 2 mmol L^{-1} EDTA, pH 8.3) as running buffer. λHind III (Gibco BRL) was used as molecular size marker.

3.8 Results and Discussion

3.8.1 Fermentation of chestnut purees

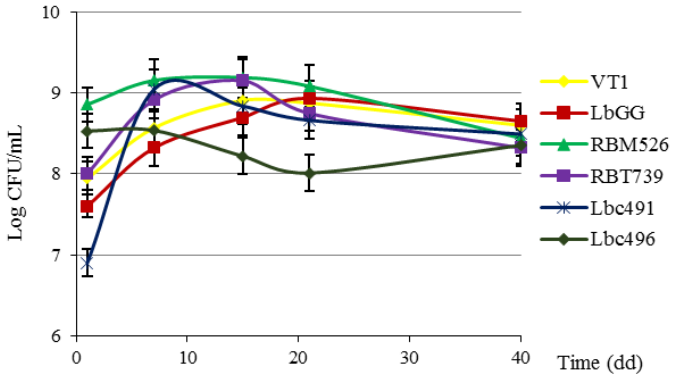
Purees prepared by broken dried chestnuts (CPs) showed a total solid soluble content of 11.5°Brix and a pH value of about 5.60 (Figure 5, Control). CPs were fermented with the six different LAB strains previously grown, up to the stationary phase, in CP and alternatively in MRS media. When the pre-inoculum was done in CP a faster acidification process was recorded in respect to MRS pre-inoculum. Results of pH and microbial evolution are reported in Figure 5. During the first seven hours of incubation LAB population increase of one decade and after 24 hours all strains reach at least 10^8 CFU mL^{-1}; in detail *Lb. rhamnosus* VT1 and RBT397 reached the highest level (10^9 CFU mL^{-1}) of population, proving to be better adapted to the media. Actually, the *Lb. rhamnosus* GG strain used in this study is well known for its ability to survive and to tolerate adverse storage conditions in dairy products (Alampreese et al., 2005), fresh-cut apple (Alegre et al., 2011), maple sap (Khalf et al., 2010), and emmer beverages (Coda et al., 2011).

Figure 5 Changes in pH and microbial counts (CFU mL^{-1} and standard deviation) during fermentation of CPs by different LAB strains. Pre-cultures prepared in CP.



Populations of lactobacilli along 40 days of storage at 4°C are shown in Figure 6. Initial populations in fermented CPs ranged between 8 and 9 Log CFU mL^{-1}; and fluctuated in this range throughout storage. In other word, all tested strains appeared to have significant potential for survival in chestnut puree and high tolerance to refrigerator temperatures. Viable counts as high as 8 log_{10} CFU mL^{-1}over 40 days of storage at 4°C suggest promise as probiotic candidates for the development of a variety of functional products being above the 6 Log CFU mL^{-1} recommended for a probiotic to exert its health benefits (Laurens-Hattingh & Viljoen, 2001).

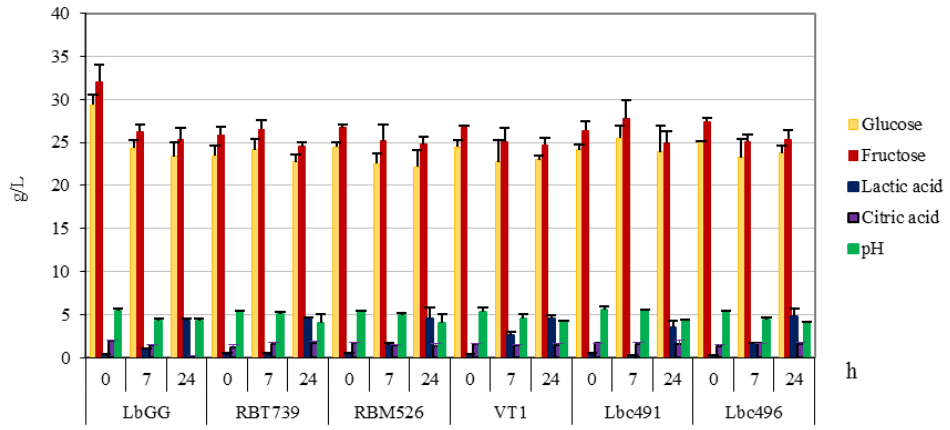
Figure 6 Evolution of LAB counts (Log CFU mL^{-1} and standard deviation) of fermented CPs during storage at 4°C.



3.8.2 HPLC analysis

Results of HPLC-based analysis for quali-quantitative determination of sugars, alcohols and organic acids are summarized in Figure 7. In the un-fermented CPs (To values, Figure 7) the concentration of free carbohydrates was about 2.7% for fructose (27.53±2.28 g L^{-1}) and 2.5 % for glucose (25.15±2.12 g L^{-1}). Citric acid (1.59±0.25 g L^{-1}) and, in minor quantity, lactic acid (0.31±0.43 g L^{-1}) were naturally present, while acetic acid was below the detection limit of the RI-detector (0.100 g L^{-1}). No significant differences among the different strains were observed in glucose and fructose utilization during fermentation (Figure 7). HPLC analysis of reducing sugars evidenced, except for *Lb. rhamnosus* GG during the first seven hours of incubation, a weak utilization of glucose and fructose. As expected, lactic acid quantity increased along the 24 h incubation with values ranging from 3.55±0.80 for *Lb. casei* Lbc491 to 4.75±0.90 g L^{-1} for strain *Lb. casei* Lbc496. On the other hand, acetic acid remained below the detection value during fermentation except for *Lb. rhamnosus* GG (0.82±0.02 g L^{-1}),thus suggesting that the heterofermentative metabolism cannot be the sole explanation. Citric acid content remained quite stable along fermentation, except for strain *Lb. rhamnosus* GG (Figure 7).

Figure7 Evolution of organic acid and reducing sugars (g L^{-1} ±standard deviation) along fermentation (data at 0, 7 and 24 h of incubation) of CPs.

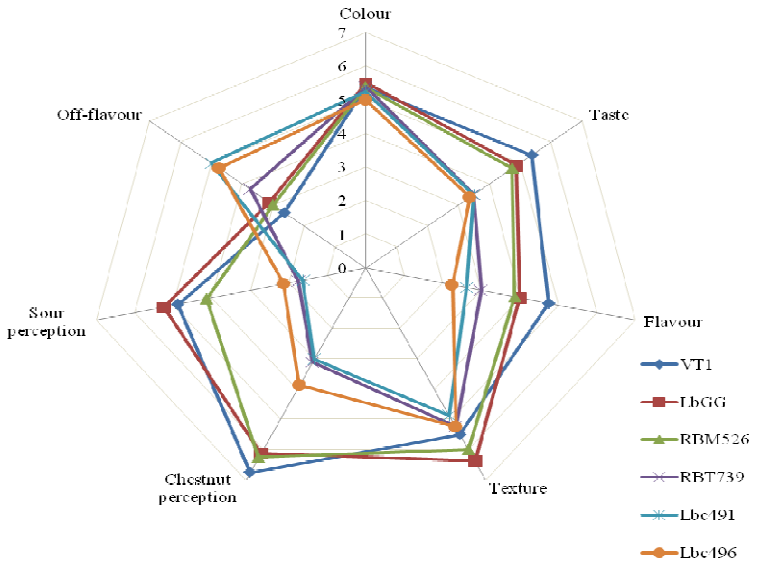


3.8.3 Preliminary sensorial evaluation

Results concerning texture and colour of purees did not show significant differences among samples (Figure 8), being both attributes quite satisfactory, with scores ranging between 5.0 and 5.5 and 4.88 and 6.38 for colour and texture, respectively (Figure 8). Noteworthy differences emerged for the attributes “chestnut perception” and “sour”. CP inoculated with strains VT1, LbGG and RBM526 exhibited a most balanced sour perception (4.2-5.2) and, as expected, this trait allowed to the CP produced with those strains to keep the highest chestnut taste (6.2-6.8) (Figure 8). Similarly, flavour and taste showed better

characteristics for samples produced with the above mentioned strains (Figure 8). As general consideration, CP inoculated with strain VT1 appeared to be the most appreciated by panelists.

Figure 8 Sensory profiles of chestnut pureeS produced by fermentation with *Lb. rhamnosus* and *Lb. casei* strains.



3.8.4 Tolerance to low pH values and to bile salts

All strains exhibited a percent of survival higher than 60% at pH 2.5 and 3.0; on the other hand at pH 2.0 just two strains (Lbc491 and LbGG) showed a percent of survival higher than 60% (Table 8). As a matter of fact, the reference pH value for this kind of test is 2.50 (Zoumpopoulou et al., 2008; Delgado et al., 2007; Kimono et al., 1999), consequently, all strains would probably be able to survive to gastrointestinal transit (GIT).All strains survived to 0.15% of bile salts, while at 0.3%, just one strain showed a percent of survival lower than 60% (Lbc491); for the remaining five strains, included the probiotic strain *Lb. rhamnosus* GG, survival rate ranged from 54.72 up to 73.27. Results from the combined trial confirm the ability of all strains to pass through the TGI: at pH 3.0 and 0.3% of bile salt, the percentage of survival ranged for all strains from 62.95 up to 92.31. On the other hand, at pH 2.0, just *Lb. rhamnosus* GG showed a percent of survival higher than 60% (70.79). Obviously, these prerequisites of probiotic interest should be even assessed in presence of pepsin and organic acids in order to ascertain the real capacity of the strains to survive to transit through the GIT.

Table 8 Tolerance to pH and bile salt, expressed as percent of survival, of *Lb. rhamnosus* and *Lb. casei* strains.

Strain	Survival percentage (% ± sd)						
	pH 2.0	pH 2.5	pH 3.0	0.15% oxgall	0.30% oxgall	pH 2.0 0.3 % oxgall	pH 3.0 0.3 % oxgall
LbGG	73.26±2.1	96.35±1.1	117.91±0.0	80.00±2.3	65.00±1.5	70.79±0.0	85.71±1.6
RBT739	35.48±0.7	70.21±0.9	98.53±1.2	88.12±1.2	73.27±0.3	45.53±3.1	78.91±1.6
RBM526	44.36±0.2	80.74±0.4	155.56±2.0	75.00±0.8	69.23±2.2	25.30±1.6	62.95±0.0
VT1	45.71±0.5	72.75±3.0	136.51±0.1	92.78±1.2	72.16±0.4	40.09±2.4	85.92±0.3
Lbc491	60.50±1.3	96.97±1.1	145.21±1.8	79.24±1.0	54.72±3.0	52.93±2.4	89.29±2.0
Lbc496	52.58±1.0	115.01±0.0	191.96±1.5	75.38±0.9	66.92±0.6	51.27±1.4	92.31±1.1

3.8.5 Susceptibility to antibiotics

All strains proved to be sensitive to amoxicillin, ampicillin, piperacillin, imipenem, rifampicin, clindamycin, and all the tested tetracyclines and macrolides On the other hand resistances were recorded for cloxacillin, oxacillin, penicillin-G, vancomycin, bacitracin, lincomycin, fusidic acid, fosfomycin, norfloxacin, novobiocin, pipemidic acid, metronidazole , sulfamethoxazole, all aminoglycosides and all cephalosporins except cephalothin (Table 9). Strains Lbc496 and Lbc491, both belonging to the species *Lb. casei* were susceptible to mezlocillin. To nitrofurantoin the sole sensitive strain was LbGG (Table 9). Actually, antibiotic resistance was even detected from Duangjitcharoenet al. (2008) in probiotic *Lb. plantarum* strains as well from Zoumpopoulou et al. (2008) in probiotic *Lb. plantarum* and *Lb. fermentum* strains. Moreover, data confirm, with very few

exceptions, results obtained by Coppola et al.(2005) and Zhou et al. (2005) for strain *Lb. rhamnosus* GG. In detail, Zhou et al (2005) reported this strain as sensitive to vancomycin, but already in 1998, Hamilton-Miller and Shah found that susceptibility to vancomycin may be useful in speciation of lactobacilli, as *Lb. acidophilus* and *Lb delbreuckii* are sensitive, while other lactobacilli, mainly *Lb. rhamnosus* are always resistant. (Hamilton-Miller & Shah, 1998).

Table 9 Susceptibility to 39 antibiotics of *Lb. rhamnosus* and *Lb. casei* strains.

Group	Name	Potency	LbGG	RBM526	RBT739	Lbc491	Lbc496	VT1
Inhibitors of cell wall synthesis								
Penicillin	amoxicillin	25 µg	MS*	S	S	S	S	MS
	ampicillin	10 µg	S	S	S	S	S	S
	cloxacillin	1 µg	R	R	R	R	R	R
	carbenicillin	100 µg	S	R	S	S	R	S
	carbenicillin	50 µg	R	S	R	R	S	R
	mezlocillin	75 µg	R	R	R	S	S	R
	Oxacillin	1 µg	R	R	R	R	R	R
	Penicillin-G	10 µg	R	R	R	R	R	R
	piperacillin	100 µg	S	S	S	S	S	S
Cephalosporins	ceftazidime	30 µg	R	R	R	R	MS	MS
	cephaloridine	30 µg	R	R	R	R	R	R
	cephalothin	30 µg	R	R	R	R	S	S
	Cefoxidin	30 µg	R	R	R	R	R	R
β lactamase inhibitors	Imipenem	10 µg	S	S	S	S	S	S
	aztreonam	10 µg	R	I	I	R	I	I
Single antibiotic	vancomycin	30 µg	R	R	R	R	R	R
	bacitracin	30 µg	R	R	R	R	R	R
Inhibitors of protein synthesis								
Tetracycline	tetracycline	30 µg	S	I	S	S	R	S
	chlorotetracycline	30 µg	S	S	S	S	S	S
	oxytetracycline	30 µg	S	S	S	S	S	S
Aminoglycosides	gentamicin	10 µg	R	R	R	R	R	R
	kanamycin	30 µg	R	R	R	R	R	R
	netilmycin	30 µg	R	R	R	R	R	I
	streptomycin	10 µg	R	R	R	R	R	I
	tobramycin	10 µg	R	R	R	R	R	R
Macrolides	erythromycin	15 µg	S	S	S	S	S	S
	spiramycin	100 µg	I	I	I	I	S	I
	oleandomycin	15 µg	I	S	S	I	S	I
Lincosamides	clindamycin	2 µg	I	I	S	S	S	I
	lincomycin	2 µg	R	R	R	R	R	R
Single antibiotic	fusidic acid	10 µg	R	R	R	R	R	R
	fosfomycin	50 µg	R	R	R	R	R	R
Inhibitors of nucleic acid synthesis								
Rimfamicins	rifampicin	30 µg	I	S	S	S	I	I
Single antibiotics	norfloxacin	10 µg	R	R	R	R	R	R
	novobiocin	30 µg	R	I	R	I	I	R
	pipemidic acid	20 µg	R	R	R	R	R	R
	metronidazole	80 µg	R	R	R	R	R	R
Sulphonamides	sulfamethoxazole	100 µg	R	R	R	R	R	R
Single antibiotic	nitrofurantoin	300 µg	S	R	R	I	R	R

* Susceptibility expressed as S (Susceptible), MS (Moderately Susceptible) or R (Resistant) according to NCCLS guidelines (2002).

3.8.6 Screening for traits of technological interest

No strain was able to express esterase activity confirming that LAB possess a weak lipolytic system (Montel et al., 1998). Similarly, no strain was able to utilize starch or citrate as the unique carbon source. Four strains, RBM526, Lbc491, Lbc491 and VT1, exhibited proteolytic activity on skim milk (Table 10). With reference to EPS production, data obtained confirmed the ability of *Lb. rhamnosus* GG strain to produce EPS and this capability was even detected in two strains belonging to the

same species (RBT739 and RBM526). *Lb. casei* strains showed a completely different behavior and similar to the *Lb. rhamnosus* strain VT1: with the sole exception of strain Lbc496, able to produce EPS from starch, the three strains did not produce EPS, independently from the employed medium. No strain expressed decarboxylase activity (Table 10). Acetoin production was evaluated by Voges-Proskauer test in four different media. Strain LbGG provided a positive response to the test in all cases, while *Lb. casei* strains, Lbc491 and Lbc496, gave a positive reaction only when MR-VP and enriched MRS broth were used as media for bacterial growth.

Table 10 Biochemical features of technological interest of *Lb. rhamnosus* and *Lb. casei* strains.

Strain			VT1	LbGG	RBM 526	RBT 739	LbG 491	LbG 496
Production	EPS from	Glucose, lactose, saccharose	-	+	+	+	-	-
		Skim milk	-	+	+	+	-	-
		Starch	-	+	+	+	-	+
		Saccharose	-	+	+	+	-	-
		Lactose	-	+	+	+	-	-
		Glucose	-	+	+	+	-	-
		APT broth	-	-	-	-	-	-
	BA from	Lysine	-	-	-	-	-	-
		Tyrosine	-	-	-	-	-	-
		Histidine	-	-	-	-	-	-
		Hornithine	-	-	-	-	-	-
Citrate metabolism			-	-	-	-	-	-
Lipolysis			-	-	-	-	-	-
Starch utilization			-	-	-	-	-	-
Proteolysis			+	-	+	-	+	-

+ Presence of an inhibition zone; - no inhibition of indicator's growth

In chestnut purees just strains LbGG and Lbc491, produced acetoin (2-butanone-3-hydroxy), which mainly confers creamy flavor (Table 11).

Table 11 Acetoin detection by Voges-Proskauer Test in four different media.

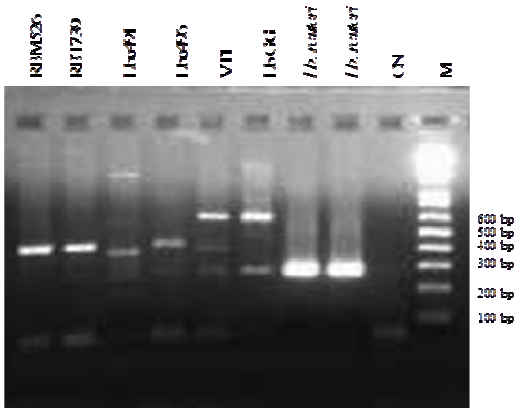
Media	**Strains**					
	VT1	LBGG	RMB526	RBT739	Lbc491	Lbc496
MR-VP	-	+	-	-	+	+
MRS	-	+	-	-	-	-
MRS + citrat	-	+	-	-	+	-
Skim milk	-	+	-	-	+	+

+ Presence of an inhibition inhibition zone; - no inhibition of indicator's growth

3.8.7 Antimicrobial activity

With the unique exception of *Listeria* spp., all strains proved to be able to inhibit, the growth of the pathogens and spoilage microorganisms used as indicators (*P. fragi, B. thermospacta, E. coli, H. alvei, S. protom, Staphylococcus spp.,* and *S. typhimurium*) (Data not shown). The contribution of acids and hydrogen peroxide productions was excluded by an assay on neutralized supernatants and by the use of the enzyme catalase, respectively. Nevertheless, each effort to prove the proteinaceous nature of the antimicrobial substances produced by bacteria failed; in fact, different proteolytic enzymes (protease, pronase, pepsin, chimopapain, α-chimotripsin and trypsin) did not reduce the inhibition activity (Data not shown). The first hypothesis was the production of a cell-associated bacteriocin. In order to spark off the release from the cells, two different approaches were employed (Jack & Tagg, 1992 and Rodríguez et al., 2010), but no activity was recorded independently by the treatment (Data not shown). As second approach, two protocols were performed in order to extract bacteriocins from agar cultures (Corsetti et al., 2003; extraction by isopropanol), but even these approaches proved to be ineffective (Data not shown). As a consequence, the ability to produce reuterin was investigated with an *in vitro* test and through the specific detection of the gene encoding for the glycerol dehydratase subunit (Figure 9). Both tests allowed excluding the reuterin production.

Figure9 PCR amplification of partial gene encoding glycerol dehydratase.

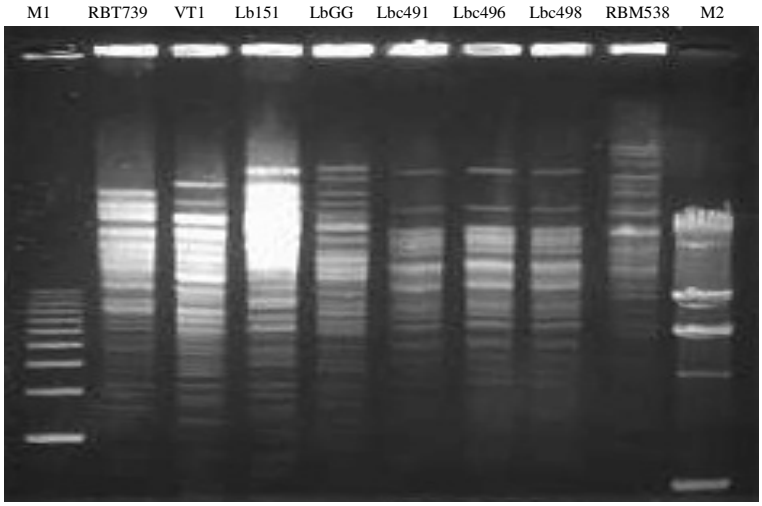


It has been previously reported that the probiotic strain *Lb. rhamnosus* GG can exerts antagonistic activity against Gram-negative pathogens (Makras et al., 2006; Fayol-Massaoudy et al., 2005, 2006; Hudault et al., 1997; Letho & Salminen, 1997). Only in a few reports was the production of organic acids suggested to be the major factor in the activity of lactobacilli against Gram-negative bacteria (Ogawa et al., 2001). In contrast, in several studies, the production of antibacterial compounds - non-lactic acid molecules **-**has been suggested to explain the activity of lactobacilli against *Salmonella* and *Escherichia coli* (Makras et al., 2006; Fayol-Massaoudy et al., 2005, 2006; Servin, 2004). Results herein presented confirm that the often recorded antimicrobial activity could be due to one or more hitherto unknown, nonproteinaceous, substances. As a matter of fact, the antimicrobial activity of probiotic strains is known to bemultifactorial (Servin, 2004), and the possibility that the mechanisms underlying LbGG antimicrobial activity against Salmonella involve both lactic acid and non-lactic acid molecules acting synergistically cannot be excluded.

3.8.8 Genetic typing of strains

PFGE of entire genomic DNA digested with *Sma*I produced complex profiles and evidenced a medium polymorphism. *Lb. casei* Lbc491and Lbc496, as well as the strain of *Lb. casei* Lbc491 coming from the same dairy environment, exhibited an identical DNA fingerprinting, in spite of the different biochemical features evidenced between the two strains (Figure 10). The detected biodiversity among *Lb. rhamnosus* strains appeared higher than the one recorded by Grzéskowiak et al. (2011); the authors, even using PFGE with two different endonucleases, failed to discriminate between *Lb. rhamnosus* GG and other strains belonging to the same species. In our study, just using one restriction enzyme, all the four analyzed strains showed different PFGE profiles.

Figure 10 PFGE profiles obtained by digestion with endonucleases *Sma*I of entire genomic DNA of the *Lb. rhamnosus* and *Lb. casei* strains.



3.9 Conclusions

This study aimed at investigating the suitability of chestnuts for making functional beverages lactose-free and with reduced fat content. Dried chestnuts were used as substrates for fermentation that was carried out using selected *Lactobacillus* spp. starters. Preliminarily, microbial analysis was carried out with the purpose to select the most suitable kind of chestnut, the proper water concentration and the technological procedure to set up fermentable purees to be used.

To our knowledge, the present study is the first to deal with the development of a probiotic product based on chestnut products. Several previous attempts have been undertaken to develop probiotic vegetable drinks for vegetarians or consumers who are allergic to dairy products (Khalf et al., 2010; Prado et al., 2008). The successful application of these innovative beverages is often restricted by the delicate nature of the ingredients, concerns over the contamination and low vitality of strains along storage. Moreover, the probiotic bacteria must be live to exert their health benefits but they can be destroyed by a number of processing situations, such as acidic conditions in the employed juices or heat treatments in a standard production. Chestnut purees proved to be the suitable media to overcome these problems.

3.10 References

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3.11 Production of an artisanal beer chestnut flavoured

The second part of a project involves the use of alcoholic fermentation. In detail a novel beer was developed by using milled barley malt and chestnut ingredients. In detail four different types of ingredients chestnut-based were evaluated: roasted dried chestnut (A), smoked chestnut flour (B), dried chestnut (C) and chestnut flour (D). Moreover two different of hops were used. The same commercial *Saccharomyces cerevisiae* were employed in all cases.

From an industrial viewpoint, the optimization of this fermentation process implicated to maximize the yeast metabolism in terms of the conversion of glucose to ethanol and the flavour extraction by chestnuts, preventing concurrently the formation of off flavours.

3.12 Materials and Methods

3.12.1 Samples and Malting and Brewing Process.

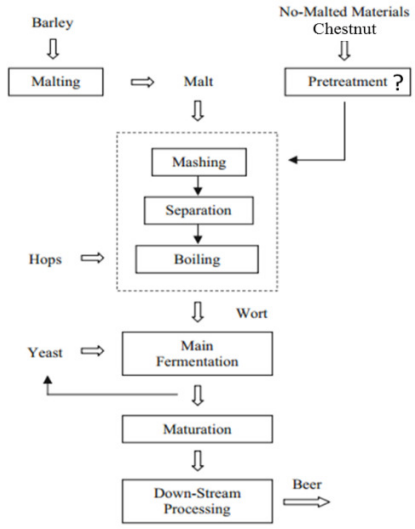
In order to evaluate how different kinds of chestnut matrices may influence the production of the chestnut beer, eight fermentations were carried on. Worts were produced according to conventional brewing techniques (Linko *et al.,* 1998) in a pilot system at the Department of Agraria, Portici, Naples. The malts used for malting and brewing were a lager-style base malt (Pilser) and two type of aromatic malt (Munich and Cara Munich II) from a commercial German malt house (Weyermann). Four different chestnut matrices (roasted dried chestnut - A, smoked chestnut flour -B, dried chestnut - C and chestnut flour - D) were combined with two different types of hops (fruity characters -1 and herbal characters - 2) (Figure 11).

Figure 11: Chestnuts, flours and hops used for the experimental production of "Beer from Montella Chestnut".

Brewing consisted of the following main processes: mashing, lautering, fermentation, and maturation. The procedure for wort production was made by using an infusion mashing schema (Figure 12).

Briefly, milled barley malt and non-malted adjuncts (chestnut) were mixed with water in a 1:4 ratio. Mashing-in took place at 52°C (15 min rest) followed by a 62°C (30 min rest) and a 71°C (30 min rest) steps; next the temperature was increased to 78°C within 15 min in order to perform the mash-out step. Mash was subsequently transferred to the lauter tun for the separation of the wort and the spent grains. Finally, the wort was boiled for 60 min at 104°C with the addition of czech (Saaz) and german (Saphir) hops. Final IBU (International Bitter Units) was setted to reach 25 units. The cooled wort (15° Brix) was pitched with yeast (Danstar Nottingham), at the rate 7×10^6 cells mL^{-1}. Fermentation was performed in stainless steel tanks (30 L). A primary fermentation was conducted at 18°C (until a 75% of attenuation) and then maturated for 30 days at 10°C (Poreda et al., 2013).

Figure 12 Technological steps for the production of the chestnut flavoured beer.



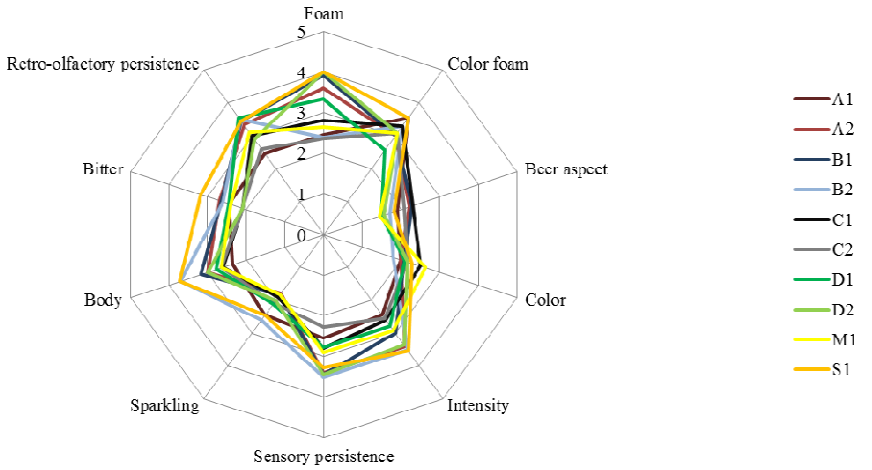
3.12.2 Sensory analysis

In order to ascertain the extent to which the selected beers, corresponding to high, moderate and low degrees of acceptability were perceived as being different from their sensory properties, a panel of twenty consumers was asked to describe the sensory profile of the products. Subjects, who participated in the sensory test only, consisted of 55% males and 45% females with an average age of 30. Ten beer samples were administered to panelists. Eight resulted from the combination of four different kind of chestnuts and two different kind of hops (A1, A2, B1, B2, C1, C2, D1, D2), one was obtained by mixing the eight beers (M1) and one was produced with smoked malts (S1). Descriptors inserted in the card were as follows: foam typology, beer aspect, sensory persistence, body, bitter, retro-olfactory persistence, alchool perception, chestnut perception and overall acceptance based on a nine-point hedonic scale ranging from 1 for "dislike extremely" to 9 for "like extremely. The control and chestnut beer samples were served in randomly numbered plastic cups and presented to the panelists at the beginning of the evaluation. Each panelist was instructed to take a few sips and then evaluate each sample for the attributes indicated on sensory ballot. Before testing the 2nd sample, each panelist was asked to take a few sips of water, eat a few bites of unsalted crackers, and drink a few sips of water to clean her palate. Panelists were also asked to write their comments on the control and chestnut beer samples. Administration conditions were kept as constant as possible, and serving temperature of the samples was restricted to range from 4 to 7°C.

3.13 Results and discussion

According to results, reported in Figure 13, relevant differences were recorded concerning taste, aroma as well as aspect. At glance, all beers proved to be characterized by the presence of creamy and dense foam and by a colour between the cream and white coffee. The liquid part was like veiled (especially in the beer made with chestnut flour), sometimes clear. The colour resulted darker for beers added of roasted chestnuts, but generally between the yellow and the mahogany. All beers presented normal intensity and persistence, with B2, D2 and S1 characterized by an higher intensity and B1 by the poorest persistence. All beers resulted poorly sparkling with a rounded form (in particular the S1 and the B2), moderate bitter taste and a sufficient retro–olfactory persistence. Chestnut taste was poorly perceived in B1 and C1 beers added of dried chestnut. According to T student test, no statistically relevant difference was highlighted between beers produced with roasted and not roasted chestnut.

Figure 13 Sensory profiles of chestnut beers produced in four different conditions.



3.14 Conclusions

According to the different kind of chestnut used and the methods with were produced, was possible to have in the product a chestnut flavour more or less intense a more or less rounded and structured form with more or less persistent foam.

3.15 References

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3.16 Chestnut employment for the development of functional and probiotic foods

Due to the tremendous increase in public concern over health issues and the diversification of food products, the functional food industry needs to explore novel processes. In accordance with the worldwide accepted definition, functional food is used to describe foods or nutrients whose ingestion leads to important physiological changes in the body that are separate and distinct from those associated with their role as nutrients (FDA, 2004).

Fermented foods containing probiotic microorganisms are generally considered as functional foods. Probiotics are defined by the FAO/WHO as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host and are widely used as a live microbial food supplement that can improve the intestinal microbial balance (FAO/WHO, 2001). Many nutritional and therapeutic benefits have been attributed to probiotic microorganisms, including metabolism of lactose, control of gastrointestinal infection, suppression of cancer, reduction of serum cholesterol, immune stimulation, and increased digestibility of foods (Collins et al., 1998). The therapeutic potential of probiotics is dependent upon a number of factors, but most important are their survival during manufacture, storage, transit through the gastrointestinal tract, and the ability to proliferate in the large intestine (Tannock, 1998). Selection of suitable strains must account for these factors (Saarela et al., 2000). The main challenges presented to probiotics, during their passage through the gastrointestinal tract (TGI), are the acidic gastric secretions of the stomach, and bile salts released into the duodenum (Bezkorovainy, 2001). Several researchers have studied the survival of microorganisms in the presence of bile salts and at low pH, and it has become clear that the survival of probiotics, both in vitro and in vivo, is strongly influenced by the food used for their delivery (Charteris et al., 1998; Dunne et al., 2001). Buffering capacity, pH, as well as physical and chemical characteristics of the food carrier have all been demonstrated to be significant factors. Foods used for the delivery of probiotics are usually dairy fermented foods (Gomes & Malcata, 1999), however probiotics are also delivered in fruit drinks, sweetened milks, infant formula and confectionary (Patel et al., 2004). Actually, chestnut appears as a functional fruit and, moreover, due to the presence of non-digestible components of the matrix, chestnut might also serve as prebiotics. However, due to the complexity of chestnut composition, a systematic approach is needed in order to identify the factors which may enhance the growth and survival of probiotic strains, both *in vitro* and *in vivo*.

3.17 Materials and methods

3.17.1 Microorganisms and culture conditions

LAB strains used in this study were as follows: *Lactobacillus* (*Lb*.) *rhamnosus* VT1, RBM526, and RBT739 isolated from Parmigiano reggiano cheese and kindly provided by the Department Agricultural, Environmental and Food Sciences (DIAAA,Campobasso, Italy), two *Lb. casei* (Lbg491, Lbg496), ten *Streptococcus* (*St*.) *thermophilus* (309, 43, 69, 82a, 80, 48, 381, 247, 75, 246) and ten *St. macedonicus* (67, 393, 72, 282, 71, 109, 335, 51, 395, 240) isolated from Provolone del Monaco cheese (Aponte *et al*., 2008); five *Lb. paracasei* subsp. *tolerans*,isolated from water kefir grains (1A, 4AM, 1S), commercial probiotic drink (1DM) and commercial probiotic tablet (4E); one *Enterococcusdurans* strain from water kefir grains (2E); two *Lb. casei* coming from probiotic drink (1B) and probiotic tablet (5EG), and, finally, one probiotic strain *Lb. rhamnosus* GG from ATCC (ATCC 53103) (Table 12). Before experimental use, cultures were propagated twice in M17 (Oxoid) for streptococci o in MRS (Oxoid) for lactobacilli and enterococci.

3.17.2 LAB strains survival under conditions simulating the human GI tract

The effect of low pH was examined by the method of Kimoto *et al.* (1999). Cells of LAB strains were harvested from MRS or M17 overnight cultures (5 mL) by centrifugation (1840 *g* for 10 min) and resuspended in 0.85% NaCl solution at pH 2.50 adjusted with 1 N HCl, and then held at 37°C for 30 min. The number of surviving cells was determined by a direct plate counting on MRS or M17 agar. Saline solution at pH 6.00 was used as control. The sensitivity of strains to bile salts was tested on MRS or M17 agar plates containing 0.30% of Oxgall (Sigma, Milan, Italy), and on the unmodified media as control; cells were harvested by centrifugation of MRS or M17 broth overnight cultures and resuspended in saline at pH 6.00. In all trials, agar plates were incubated at 30 or 37°C for 24–48 h, and the number of colonies counted. Each experiment was performed in triplicate.

3.17.3 Preparation of chestnut media and chestnut extracts

Chestnut flour from cv “Castagna di Montella” was used for the preparation of culture media. 5 g were mixed with 95 mL of distilled water under heating at 80°C for 20 min and sterilized at 121°C for 20 min before use as chestnut media (CM). The chestnut extract (CE) was prepared by centrifuging (5000 rpm, 10 min) CM to separate solids. Chestnut flour (protein: 4.61%; lipid: 3.8%; carbohydrate: 69.3%) was kindly provided by Ipafood srl (Avellino, Italy).

3.17.4 Preparation of free cells and cells immobilized within chestnut fiber

Strains were grown overnight in MRS or M17 medium, for lactobacilli (incubation at 30°C) and streptococci (incubation at 37°C), respectively; transferred (1%, v/v) to fresh medium or chestnut media, and cultured for a further 24 h. The free cells were prepared using the following procedure: overnight broth cultures were centrifuged (5000 rpm, 10 min), the pellets washed

twice with sterile saline (0.5%, w vol^{-1}), and the cells resuspended in sterile saline (0.5%, w vol^{-1}). The cells immobilized within chestnut fiber (CF) were prepared as follows: cells cultured in CM were sedimented and washed twice using sterile saline (0.5%, w vol^{-1}) and resuspended in sterile saline (0.5%, w vol^{-1}).

3.17.5 Preparation of simulated gastric and bile juice

Simulated gastric and bile juices were prepared daily. Simulated gastric juices were prepared by suspending pepsin (1:10 000, ICN, Sigma) in sterile saline (0.5%, w/v) to a final concentration of 3 g L^{-1} and adjusting the pH to 2.00 with concentrated HCl using a pH meter. Simulated bile juices were prepared by suspending pancreatin USP (P-1500, Sigma) in sterile saline to a final concentration of 1 g L^{-1}, with 4.5% bile salts (Sigma) and adjusting the pH to 8.00 with sterile 0.1 mol L^{-1} NaOH.

3.17.6 Measurement of gastric and bile tolerance

The tolerance of LAB to the simulated gastric and bile juices was determined according to the procedure reported by Michida et al. (2006). Briefly, aliquots (0.2 mL) of the free-cell suspensions from the MRS or M17 medium and of cells immobilized within chestnut fiber were separately transferred to a 2.0 mL capacity Eppendorf tube, mixed with 0.3 mL of sterile saline (0.5%, w/v) or CE (5 %, w/w), and finally mixed with 1.0 mL of simulated gastric (pH 2.00) or bile juice (pH 8.00). The mixture was then incubated at 37°C. Aliquots of 50 µL were taken after 0, 15, 30, 60, 90, and 180 min and after 0 and 240 min for gastric and bile tolerance measurements, respectively.

Table 12 Tolerance to low pH and bile salts, expressed as percent of survival (% ± sd), of strains used in this study.

Taxon	Source	Strain	Survival percentage (% ± sd)	
			pH 2.50	0.30% oxgall
St. macedonicus	Provolone del Monaco cheese	67	6.80±1.55	30.80±1.36
		393	78.79±0.39	60.61±1.07
		72	48.82±1.74	41.76±1.45
		282	40.00±1.13	11.20±0.58
		71	8.71±2.37	1.18±0.95
		109	9.13±1.04	46.09±0.83
		335	95.04±0.52	99.96±0.53
		51	17.65±0.38	0.90±0.76
		395	45.95±1.05	22.16±1.06
		240	5.79±1.24	1.01±1.34
St. thermophiles		309	7.41±0.80	28.70±0.64
		43	19.07±0.22	0.43±0.94
		69	54.55±0.83	49.09±1.13
		82a	3.84±0.51	13.95±0.72
		80	64.10±1.77	12.76±2.55
		48	19.21±4.00	15.39±4.91
		381	10.29±0.80	0.10±0.74
		247	100.67±0.37	3.17±0.22
		75	15.75±1.27	4.75±0.84
		246	22.22±1.68	3.53±0.68
Lb. paracasei subsp. *tolerans*	Water kefir grains	1A	54.78±0.51	33.91±0.76
		4AM	16.95±1.43	54.24±1.39
		1S	99.90±2.24	19.52±2.24
Enterococcusdurans		2E	0.27±1.14	10.24±0.63
Lb. casei	Probiotic drinks	1B	21.43±1.07	60.95±1.30
Lb. paracasei subsp. *tolerans*		1DM	100.00±0.66	100.94±0.80
Lb. paracasei subsp. *tolerans*	Probiotic tablets	4E	64.65±0.39	100.42±0.63
Lb. casei		5EG	42.55±0.64	1.06±1.26

3.17.7 Viable cell counts

The drop method (Collins et al., 1989) was used to determine gastrointestinal viability. Samples diluted (10^{-1} to 10^{-6}) with sterilized Ringer's solution (Oxoid) were prepared for cell counts. Five aliquots of 12 µL were dropped onto MRS agar plates using a precalibrated 20 µL micropipette. The plates were incubated at 30 or 37°C for 48 h and those showing individual colonies in the drop areas were counted. Viable cell counts were calculated as colony-forming units per mL and the results expressed as Log10 values.

3.17.8 Analysis of reducing sugars and peptides composition by HPLC

HPLC analysis of reducing sugars was performed on samples diluted in ultrapure water 1:10 (v/v) and filtered by AcroDisc millipore (0.02 μm) by system (Gilson 307 Series HPLC system) fitted with column MetCarb 68H column in an oven thermostated at 65°C (Blaiotta et al., 2012). Columns were eluted at 0.4 mL/min by a solution 0.25 N H_2SO_4 in ultrapure water. A refractometer (RID 133, Gilson) was used as detector. Standards were used for quantification of different sugars and acids in the samples. Each experiment was performed in triplicate.

Reversed phase HPLC analysis of peptides was performed on filtered aqueous extract using a 250 x 460 mm C_{18} (5 μm, 110 Å) Chemetex column, according to procedure detailed by Desportes et al., (2000) modified as follows: Column was eluted at 0.5 mL min^{-1} by a linear gradient of two eluents: A (0.1% Trifluoroacetic acid in water) and B (0.1% Trifluoroacetic acid in acetonitrile). A linear gradient was developed from 0 to 51% of eluent B over 65 min, from 51 to 100% of eluent B over 1min, from 100 back to 0 % of eluent B in 3 min and, finally, 5 min for column re-equilibration. The elution was detected by a diode array at 220 nm in order to have an approximate recording of the peptide bond that was better evaluated by UV spectra for any peaks between 40 and 50 min of elution. Fractions were collected every 10 min. To establish at what percentage of acetonitrile, the component responsible for strains' gastric tolerance was eluted, chromatograms of CE from cv "Castagna di Montella" were divided in six fractions. Each fraction was freeze-dried, dissolved up to 300 μL in sterilized water and tested for the ability to protect strain LbGG in gastric juice according to the protocol previously detailed. Counts by drop method for each fraction were carried on at time 0 and after 90 and 180 min of exposure. Each experiment was performed in triplicate.

3.17.9 Influence of the kind of chestnut flour on strains' tolerance to gastric conditions

Chestnut media and Chestnut extract were even prepared using flours obtained from milling of chestnuts produced in Emilia Romagna region (North Italy) and Calabria region (South Italy). In detail, chestnut flour from Northern Italy (protein: 7.05%; lipid: 6.94%; carbohydrate: 60%) was purchased from "Molino Spadoni S.p.A." (Ravenna), whereas flour from Southern Italy (protein: 6.1%; lipid: 3.7%; carbohydrate: 76.2%) was purchased from "Antichi Sapori" (Catanzaro). Tests to assess microbial viability during transit in simulated gastric juice were performed as previously described by using strain LbGG.

3.17.10 Influence of the protocol employed for CE production

The potential influence of the protocol adopted to obtain CE was evaluated. In detail, CEs were prepared by mixing flour and water at 20, 53 and 80°C and used for the experiments as above detailed, after centrifugation and filtration through a 0.22 μm-pore-size cellulose acetate filter (Sartorius, Goettingen, Germany). Moreover, CEs were prepared by heating at 80°C for 5, 10, 15 and 20 minutes under stirring, and then centrifuged and filtered. Finally, CEs were prepared according to the conventional procedure (80°C for 20 min, sterilization in autoclave) but followed by centrifugation at 14.000 rpm for 10 min, instead of 5000 rpm.

3.17.11 Effect of reducing sugars and soluble starch on strains' gastric tolerance

The influence of starch was evaluated by preparing extracts to be assayed with commercial potato flour and soluble starch (Sigma). Extracts were prepared following the same protocol developed for CE. The contribute of reducing sugars was ruled out by experiments performed, substituting CE with sugar solutions whose composition was established according to data derived by HPLC analyses. In detail, three sugar solutions were tested: fructose (9 g L^{-1}) and glucose (8 g L^{-1}), only maltose (2 g L^{-1}) and all of them at the same concentrations here reported.

3.17.12.Heat challenge experiments

The thermal tolerance of strains was compared in three different conditions: (i) cells in stationary phase of growth from MRS were harvested by centrifugation (8160 g, 10 min), washed in Ringer's solution (Oxoid) and resuspended, at the same ratio, in CE (around 4% solid content) or alternatively (ii) in filter-sterilized trehalose (Sigma) solution (20% wt vol_1); (iii) cells in stationary phase of growth from CM were collected by centrifugation, washed and resuspended in CE (1 : 1). In all cases, samples (2 ml) were put into sterile A-R glass capillaries (Kleinfeld Labortechnik GmbH, Gehrden, Germany) and placed in a water bath at the test temperatures of 60, 65 and 70°C. At 30-s intervals (0, 30, 60, 90, 120, 150, 180, 210 and 240 s), capillaries were removed and cooled in ice bath, after which a sample volume of 100 μl was serially diluted in Ringer's solution and drop-plated on MRS agar (Collins et al. 1989). Survivors were enumerated after 48 h of anaerobic incubation at 37°C (AnaerogenTM, Oxoid).

3.17.13 Drying process

The spray-drying process of *Lb. rhamnosus* strains was undertaken in a laboratory scale spray dryer (B-191; Büchi, Milan, Italy). Strains were grown in MRS medium (1:l) at 37°C up to the stationary phase. After centrifugation, cells were washed with Ringer's solution and resuspended (1:1) in CE (about 10^9 CFU g^{-1}). The feed solution was pneumatically atomized into a vertical, co-current drying chamber using a two-fluid nozzle (0.7 mm). The outlet temperature was adjusted from 60 to 75°C by varying the flow rate. The following combinations inlet/outlet temperatures were evaluated: 140/60°C, 140/65°C 140/70°C and

130/65°C. The dried powders were collected in a product container connected at the bottom of the single cyclone separator.

3.17.14 Determination of moisture content of dried samples
The residual moisture content of spray-dried samples was determined by oven-drying the powders at 105°C, determining the difference in weight and expressing the weight loss as a percentage of the initial powder weight (International Dairy Federation Standard 1993).

3.17.15 Enumeration of lactobacilli after spray drying
To determine the survival rate of the strains, 1 g spray-dried powders were rehydrated (1:10) in sterile Ringer solution, serially diluted and plated in duplicate on MRS agar. Plates were incubated anaerobically (Anaerogen TM) at 37°C for 48 h, a period at which countable colonies were observed.

3.17.16 Sensitivity test
To quantify the cellular damage arising from the spray-drying process, the sensitivity of cultures to NaCl, before and after drying processes, was evaluated (Gardiner et al. 2000). To select the adequate NaCl concentration, the minimum inhibitory concentration (MIC) was determined, and sub-inhibitory concentration was used. To assess MIC, strains were grown in MRS broth with increasing salt contents (4, 5, 6, 7, 8, 9, 10 and 12%). After 24 h of incubation, O.D. values were measured at a wavelength of 595 nm and compared with cultures in MRS without NaCl. Not fatally injured cells were thus evaluated by plating on MRS agar plates supplemented with 8% NaCl. The plates were examined after 3 and 5 days of anaerobic incubation, and viable numbers were compared with numbers obtained by counting on MRS without NaCl.
On samples processed in the best conditions, namely at a constant air inlet temperature of 140°C, outlet temperature of 65°C and flow rate of 4.5 ml min^{-1}, bacterial viability per gram was assessed using in parallel: direct counting at microscope in Petroff-Hausser chamber (total cells), live/dead® BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, OR) followed by epifluorescence microscope observation, plating onto MRS agar after incubation at 37°C for 48 h (living cells) and MRS agar supplemented with 8% after 5 days of incubation NaCl (not fatally injured cells). For the fluorescence microscopy test, cells were dyed using two fluorochromes SYTO 9 (green dye: undamaged cells) and propidium iodide (red dye: dead or injured cells). After staining followed by incubation in the dark for 15 min at room temperature, samples were observed using a Nikon Eclipse E400 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a UV lamp and a 9 100 magnification objective. Images were captured by a Nikon Coolpix 4500 Digital Camera equipped with a microscope adapter. To evaluate the survival to simulated gastric juice, powders (0.5 g) were resuspended (1/1 wt vol^{-1}) in sterile saline solution and added of simulated gastric juice (1 mL); afterwards, the same procedure detailed above was repeated.

3.17.17 Production of chestnut mousse
3.17.17.1 Ingredients for mousse
Chestnut flour from cv 'Castagna di Montella', as well as ingredients for chestnut mousse, was kindly provided by Ipafood srl company (Frigento, Avellino, Italy). Components used in the preparation of laboratory mousses are listed in Table 13.

Table 13 Composition of the anhydrous basis for chestnut mousses.

Ingredients	g Kg^{-1}
Chestnut flour	300
Hydrogenated coconut fat	150
Cocoa powder	100
Sucrose	150
Maize starch	100
Modified starch	30
Milk proteins	50
Potato starch	10
Glucose syrup	40
Mono- and diglycerides of fatty acids	15
Carrageenan	40
Guar gum	15

3.17.17.2 Mousse- making procedure
Pilot-scale anhydrous formulations for chestnut mousses were produced in triplicate in collaboration with Ipafood. Spray-dried cultures in CE of each tested strain were separately added to the mousse powder (1% wt wt^{-1}) to obtain concentrations of approximately 9 log CFU g^{-1} of living and undamaged cells in the final product. In detail, three mousse bases were produced:

probiotic (P) supplemented with strain LbGG, functional (F) supplemented with strain RBM526 and control (C) without bacterial strains. Samples were weighed, packaged in heat-sealed plastic aluminium foil bags (35 g each) and stored at 15±1°C for up to 120 days. Viability of lactobacilli in chestnut mousses P and F was monitored during the storage in two replicate trials. Monthly, samples were rehydrated with sterile deionized water (100 mL 35 g^{-1}), and viable counts were carried on using MRS agar. All samples were made from the same model cream. For preparation, 35 g of mousse powder and 100 mL of cold milk were mixed in a planetary kneader (KitchenAid KSM150PSMC Artisan, Denver, CO), equipped with a 4.800-L bowl, on medium speed for 1 min, followed by 2 min at high speed. After chill at 4°C for 2 h, chestnut mousse was ready to be consumed. Each lot of chestnut mousse was produced in amounts to obtain 2–3 kg of the final product.

3.17.17.3 Descriptive sensory analysis

Chestnut mousse samples (trials C, P and F) were analysed by a descriptive panel of 10 expert assessors (seven males and three females, aged between 25 and 55 years) with experience in sensory analysis (UNI 10957 2003). Samples (40 ml) were presented in white plastic cups. Unsalted biscuits and warm water were used to rinse mouth between evaluations of the samples. The judges were trained in three preliminary sessions using different samples of commercial chocolate mousses, to develop a common vocabulary for the description of the sensory attributes of chestnut mousses samples and to familiarize themselves with scales and procedures. The judges generated sensory terms individually. Each attribute term was extensively described and explained to avoid any doubt about the relevant meaning. Finally, sixteen attributes were selected by consensus (the frequency of citation >60%) to describe the mousse sample: intensity of the brown colour, compactness (Aspect); odour intensity, cocoa odour, vanilla odour, chestnut odour (Odour); cocoa flavour, chestnut flavour, taste intensity, sweet, astringency, bitter, bitter aftertaste (Gustative sensorial profile); texture, creamy, melting (Tactile) and overall impression. The panelists used a 10-cm structured scale to rate the intensity of each attribute. Random samples were evaluated by assigning a score between 1 (absence of the sensation) and 9 (extremely intense) in individual booths under incandescent white lighting at the sensory laboratory of the CAISIAL (Portici, Naples). A computerized data collection program was used (FIZZ, Software Solutions for Sensory Analysis and Consumer Tests, Biosystemes, Couternon, France). The mean of 10 evaluations was reported.

3.17.18 Statistical analyses

Statistical analysis was performed using SPSS, version 17.0 (SPSS Inc., Chicago, IL). Analysis of variance was carried on in order to evaluate differences in physicoechemical parameters among chestnut extracts obtained by different kinds of flours. Significant differences between the detected parameters were compared by means of Duncan's multiple comparison test at the 95% confidence level ($p \leq 0.05$). The Duncan's multiple comparison test was applied also to discriminate amongst the means of descriptive sensory data at the 95% confidence level ($p \leq 0.05$). Results were expressed as means and standard deviation of at least three independent experiments.

3.18 Results and Discussion

3.18.1 Tolerance to low pH values and to bile salts

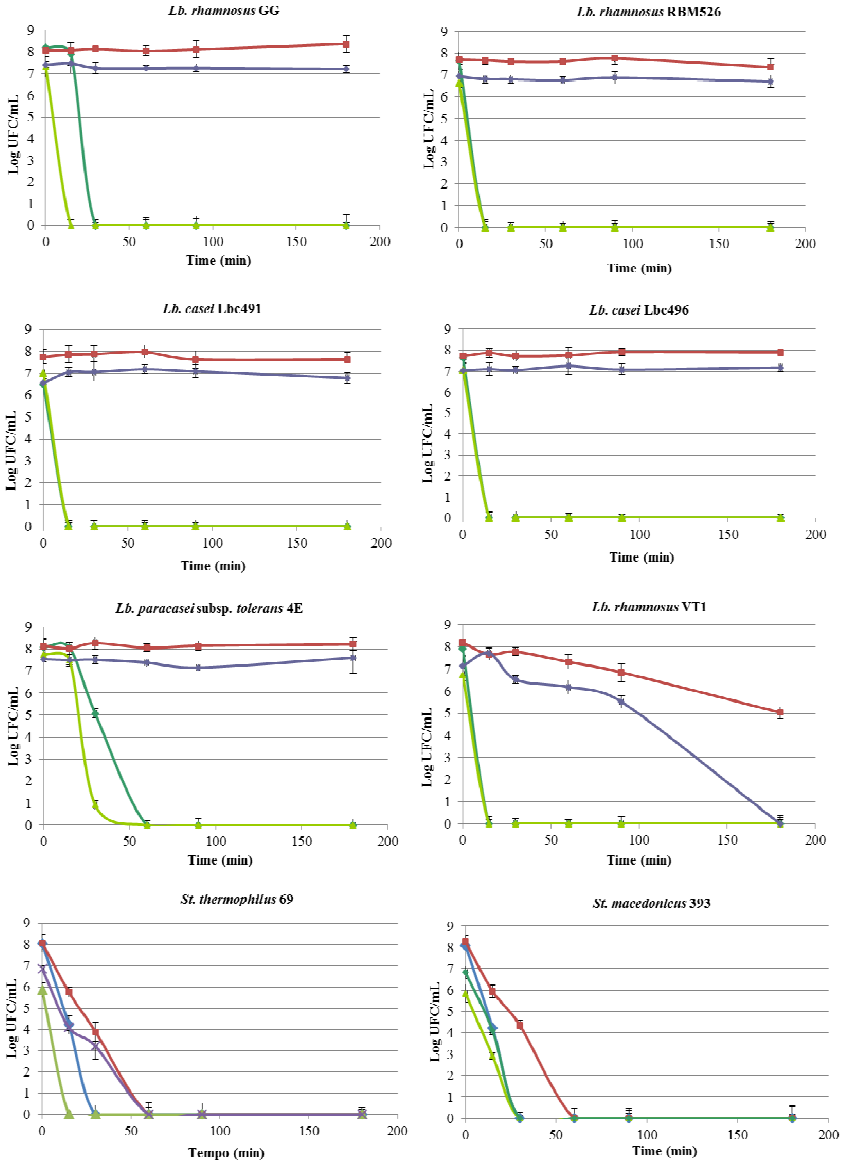
Tolerance to low pH values and to bile salts was evaluated for all strains except for *Lb. rhamnosus* VT1, RBM526, RBT739 and LbGG, as well as for *Lb. casei* Lbc491 and Lbc496, that were screened for such traits during a previous survey (Blaiotta et al., 2012). Data concerning the remaining 28 strains are reported in Table 12. Among *Streptococcus* strains, only four strains out of 20, exhibited a percent of survival higher than 60% at pH 2.50, as well as in presence of bile salts (Table 12). No strain coming from water kefir grains proved to be able to bear environmental conditions of TGI. Moreover, both *Lb. casei* strains (1B and 4 EG), even if isolated from probiotic products, did not show a proper viability under the tested conditions (Table 12). By combining results, apart from the above mentioned five promising probiotic candidates and LbGG, six strains were chosen, namely, two *Lb. paracasei* subsp. *tolerans* (1DM, 4E), two *St. thermophilus* (69, 80), and two *St. macedonicus* (335, 393). Actually, these last two species, above all *St. thermophilus* did not prove to be really able to bear environmental conditions of GIT. Nevertheless, it must be stressed that the species *St. macedonicus*, even being GRAS (Maragkoudakis et al., 2009), has never being study for its potential as probiotic.

3.18.2 Effect of CE and of cells immobilization in chestnut fiber on gastric tolerance

Survival of free cells cultured in MRS or M17 medium and cells immobilized within chestnut fiber was evaluated during transit in simulated gastric juice. CE proved to be able to exert a significant effect on the simulated gastric tolerance of free cells, as well as of cells immobilized within chestnut fiber (Figure 14). All *Lactobacillus* spp. species, with the unique exception of *Lb. rhamnosus* VT1, showed an identical behavior: population levels remained unchanged up to 180 min. in presence of CE, independently by immobilization in chestnut fiber. Actually, population levels reached by strains in MRS were about one log higher than levels in CM (Figure 14). The protective effect of CE did not appear in strains belonging to the genus *Streptococcus* spp. On the other hand, immobilization in CF did not seem to exert any beneficial effect on strain surviving. In all cases, except

Lb. casei 4E and *Lb. rhamnosus* LbGG, population levels decline under the detection level after 15 min of treatment. Data for selected representative strains are reported in Figure 14.
The obtained results appear significantly different from those reported by Michida et al. (2006). The authors evaluated the effect of cereal extracts obtained from barley and malt on a probiotic *Lb. plantarum* strain in a similar set of experiments. According to evidences they reported an absolute tolerance of strain to gastric acidity only when cells were immobilized in cereal fiber. Results obtained in the present survey appeared even conflict with those reported by Charalampopoulos et al. (2003). Results presented in their study suggested that malt, wheat and barley extracts exert a protective effect on *Lb. plantarum*, *Lb. acidophilus* and *Lb. reuteri* viability under acidic conditions, and that the phenomenon could be mainly attributed to the presence of soluble sugars in the cereal extracts, and to a less extent to the free amino nitrogen content (Charalampopoulos et al., 2003).Of course, pH was measured after simulated gastric juice adding in all cases, and no significant change was recorded (data not shown). Data for selected representative strains are reportedin Figure 14.

Figure 14 Viability of free and immobilized within chestnut fiber cells during 180 min of incubation in presence of simulated gastric juice, with and without CE. Values are means of three determinations (±sd) calculated with 95% confidence. Experimental data symbols: (◆) free cells in saline solution, (■) free cells in CE, (□) immobilized cells in saline solution; (◆) immobilized cells in CE.



3.18.3 Effect of Chestnut extract and of cells immobilization in chestnut fiber on bile salts tolerance

Strains tolerance to bile salts exhibited a completely differ trend. All tested strains, included streptococci, were able to survive up to 240 min (Table 14). In some case at the end of monitoring counts were slightly higher than those recorded at the beginning. One exception can be reported for strains *Lb. rhamnosus* RBM526 and RBT739, and for *Lb. casei* Lbc496: free cells in saline solution at the end of the treatment were about two logs lower (Table 14). As general consideration, chestnut-fiber-entrapped cells showed higher stability in the presence of simulated bile juice than free cells cultured in MRS medium.

Table 14 Tolerance of LABstrains, free cells (FC) or immobilized within chestnut fiber (IC) to simulated bile juice and effect of chestnut extract (1%) on viability at 0 min and 240 min. Values expressed as Log CFU ± sd.

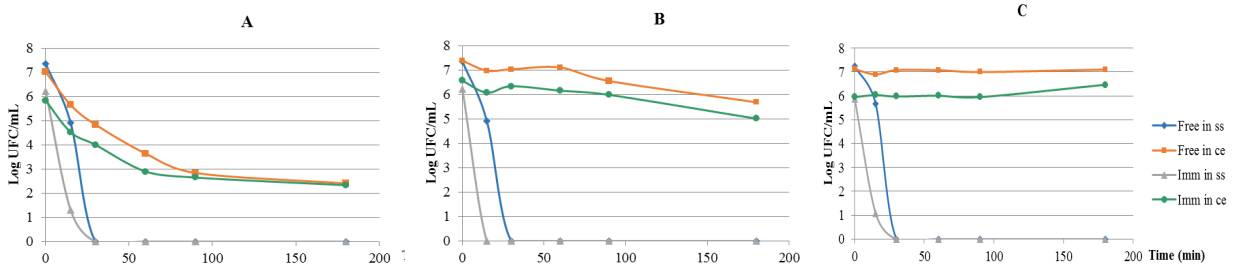
Strain	LbGG		VT1		RBT739	
Time (min.)	0	240	0	240	0	240
FC in SS[a]	7.14±0.56	7.99±0.15	8.04±0.89	7.87±0.26	7.73±0.67	5.87±0.08
FC in CE	8.07±0.45	8.20±0.77	8.06±0.34	8.73±0.50	7.81±0.66	7.95±0.54
IC in SS	6.81±0.76	7.28±0.63	6.35±0.57	6.32±0.81	7.51±0.49	7.18±0.37
IC in CE	6.56±0.92	6.97±0.41	6.56±0.54	6.88±0.76	7.20±0.25	7.56±0.93
Strain	RBM526		Lbc496		Lbc491	
Time (min.)	0	240	0	240	0	240
FC in SS	7.80±0.05	5.50±0.13	7.90±0.02	5.75±0.21	7.95±0.06	7.77±0.16
FC in CE	7.82±0.08	7.92±0.48	7.88±0.76	7.65±0.44	8.03±0.36	7.97±0.07
IC in SS	7.03±0.06	7.08±0.42	7.19±0.22	7.22±0.56	7.47±0.06	7.09±0.02
IC in CE	7.05±0.16	6.44±0.16	8.13±0.23	7.23±0.11	8.68±0.18	7.16±0.16
Strain	4E		80		335	
Time (min.)	0	240	0	240	0	240
FC in SS	7.44±0.08	8.22±0.18	8.05±0.16	8.12±0.04	8.49±0.14	8.38±0.16
FC in CE	8.07±0.21	8.35±0.32	7.88±0.04	8.28±0.10	8.20±0.04	8.40±0.60
IC in SS	8.21±0.12	7.43±0.01	7.06±0.16	8.18±0.08	7.30±0.06	8.22±0.12
IC in CE	7.19±0.18	7.8 3±0.30	7.06±0.14	8.37±0.33	7.22±0.71	8.44±0.36

[a]SS: saline solution; CE: chestnut extract.

3.18.4 Influence of the kind of flour on the efficiency of the chestnut extract

To understand if the recorded effect in strains' protection under conditions simulating the gastric transit could be related to the kind of chestnut flour employed for the preparation of CE and CF, the same set of experiments was repeated by using two flour obtained by chestnut of different origin (Flour from Northern Italy A and flour from Southern Italy B). All analyzed strains exhibited a different behavior and even lactobacilli were unable to exert the same performances (Figure 15). Cell immobilized in CF and in presence of CE of strain LbGG did not survive to an exposition 30 min long, if CE was produced by chestnut flour coming from Emilia Romagna region (Graphic A, Figure 15). A better performance was recorded when flour from Calabria region was employed (Graphic B, Figure 15); nevertheless, the tolerance expressed by LbGG strain in presence of CE manufactured with flour produced by chestnuts of cv "Castagna di Montella" remained unmatched (Graphic C, Figure 15).

Figure15 Viability of free and immobilized within chestnut fiber cells of strain LbGG during 180 min incubation in presence of simulated gastric juice with and without CE. CE and CF were produced by using flour from Emilia Romagna region (A), Calabria region (B), cv "Castagna di Montella" (C). Values are means of three determinations (±sd) calculated with 95% confidence.



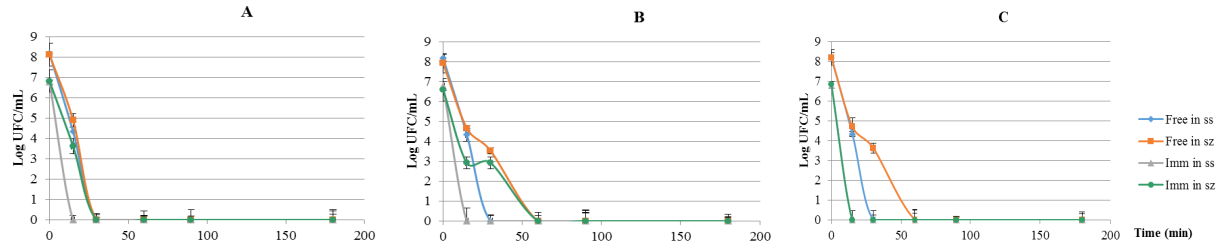
3.18.5 Chemical analysis of CEs and evaluation of the influence of reducing sugars in strains' gastric tolerance

A quali-quantitative characterization of reducing sugars was obtained by HPLC for CEs obtained by the three flours employed in the previously described experiment (Table 15). Glucose, fructose and maltose were the only sugars retrieved in all cases, but their contents were different (Table 15). CE from cv "Castagna di Montella" resulted richer in sugars and above all in maltose, whose content was almost three times higher (1.79 g L^{-1}) if compared to the values obtained for the other two analyzed CEs. Experiments were repeated by substituting CE with solutions containing three different combination of sugars as derived by HPLC analysis of flour by cv "Castagna di Montella". Results, reported in Figure 16 for strain LbGG, allowed to exclude the contribution of reducing sugars in the improving of gastric tolerance of lactobacilli.

Table 15 pH, soluble solid content and concentration of reducing sugar as revealed by HPLC analysis of CEs produced with flours of different geographical origin. Means with different letters are significantly different ($p< 0.05$).

Parameter	Composition of CEs produced with flour from:		
	Emilia Romagna region	Calabria region	cv "Castagna di Montella"
pH	$5.43\pm0.20_a$	$5.58\pm0.17_a$	$5.75\pm0.20_a$
°Brix (°Bx)	$3.50\pm0.11_a$	$3.83\pm0.22_b$	$4.00\pm0.12_b$
Glucose (g L^{-1})	$6.65\pm0.38_a$	$7.01\pm0.15_a$	$7.84\pm0.45_b$
Fructose (g L^{-1})	$6.60\pm0.45_a$	$6.84\pm0.12_a$	$8.61\pm0.49_b$
Maltose (g L^{-1})	$0.47\pm0.22_a$	$0.63\pm0.14_a$	$1.79\pm0.17_b$

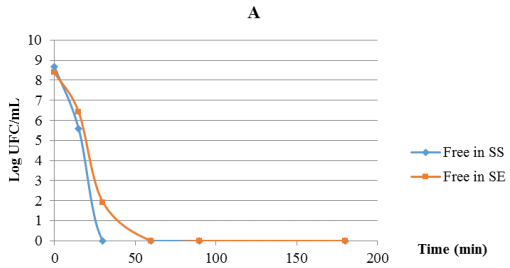
Figure 16 Viability of free and immobilized cells of strain LbGG during incubation in simulated gastric juice in presence of solutions containing fructose (9 g L^{-1}), glucose (8 g L^{-1}) and maltose (2 g L^{-1}) (A); fructose (9 g L^{-1}) and glucose (8 g L^{-1}) (B); maltose (2 g L^{-1}) (C). Values are means of three determinations (±sd) calculated with 95% confidence.



3.18.6 Effect of starch on the strains tolerance to simulate gastric juice

In order to highlight a potential implication of soluble starch in the expression of the recorded gastro-protective effect associated to CE, experiments were repeated by preparing extracts with two different starchy matrices: potato starch and soluble starch. Results showed that both extracts did not guarantee the same performance in the level of tolerance of the strain LbGG, as highlighted in the presence of CE. In both case, already after 30 min. of exposure to simulated gastric juice, the population level declined, dropping below the detection threshold of the counting method after only 60 minutes (Figure 17).Likely, the recorded protective effect of CE on microorganisms in simulated gastric juice could not be simply related to the high content of starch in the flour obtained from the milling of the chestnut as inferable by test performed with starchy matrices.

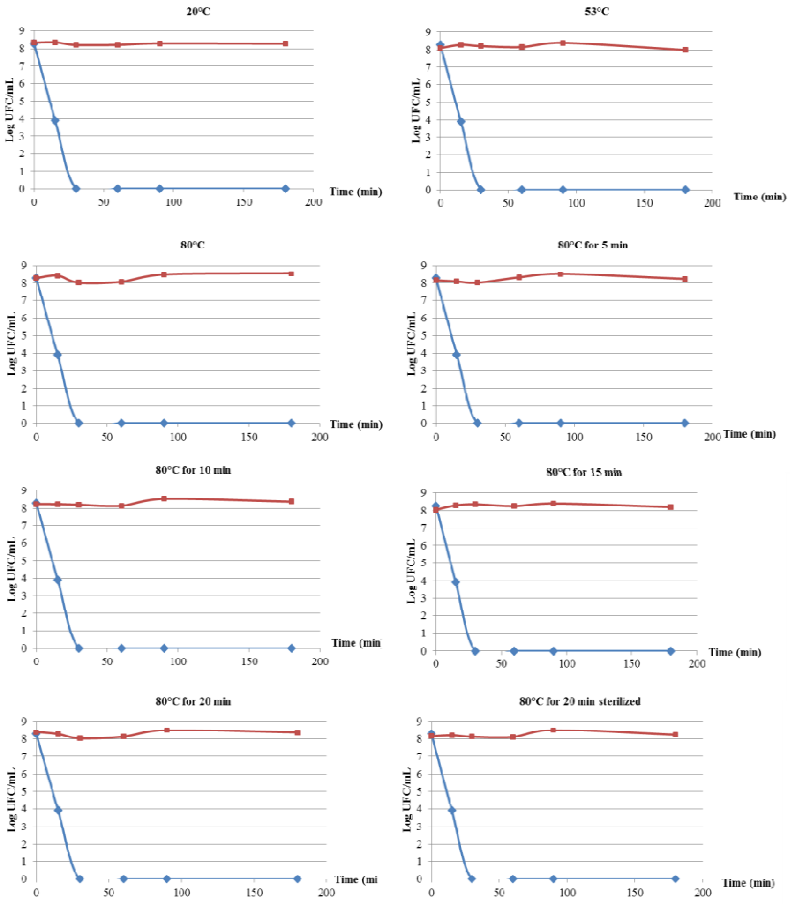
Figure 17 Viability of free cells of strain LbGG during 180 min incubation in presence of simulated gastric juice with and without two different starchy matrices. SE and PF were produced by using soluble starch (A), potato starch (B). Values are means of three determinations (±sd) calculated with 95% confidence.



3.18.7 Effect of the preparation protocol on CE efficiency

To verify whether the exposure to high temperatures and the speed of centrifugation, during CE production, could exert any positive effect, the protocol was modified in eight different ways. According to results, the mode of preparation of CE does not express any influence on the protective properties of the same during gastric transit (Figure 18). The sole cold mixing of flour in water allows obtaining a CE able to guarantee the previously recorded strains' resistance to simulated gastric juice for the entire period of exposure, by stabilizing the population level to the initial values (about 10^8 CFU mL^{-1}).

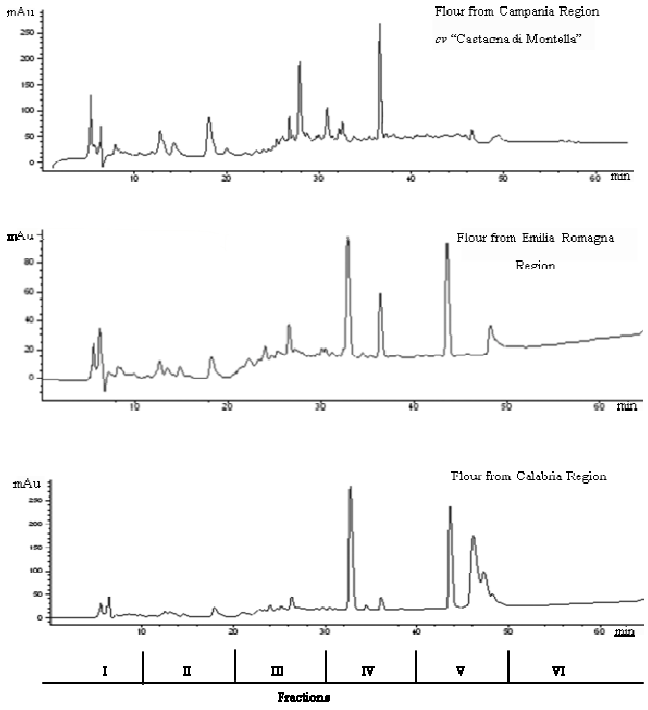
Figure18 Viability of free cells of strain LbGG, during 180 min incubation in simulated gastric juice, using different protocol for chestnut extract production.Experimental data symbols (■) free cells in saline solution, (◇) free cells in CE



3.18.8 Peptides composition by HPLC

Chromatogram profiles obtained by RP-HPLC of the three CEs are reported in Figure 19. As clearly evident, CEs obtained from chestnut flours of different origin exhibited completely different profiles. Six fractions from CE by cv "Castagna di Montella" were tested for the ability to protect LbGG during gastric transit. The initial population level of the strain was 9.08±0.18 Log CFU mL^{-1}. In detail, no growth was recorded in fractions I, II and VI at the beginning of monitoring as well as at 90 and 180 min (Data not shown). On the other hand, in fractions III, IV and V, the recorded initial population levels were 9.14±0.29, 7.03±0.35 and 8.92±0.03 Log CFU mL^{-1}, respectively. After 90 min of treatment, microbial loads collapsed in all cases, declining to zero in case of fractions III and IV and to 4.22±0.04 Log CFU mL^{-1} forfraction V (Data not shown). By contrast, after 180 min, an higher population level was detected, with counts ranging from 3.99±0.02 and 3.88±0.11 Log CFU mL^{-1} for fractions III and IV up to 6.22±0.04 Log CFU mL^{-1} for fraction V (Data not shown). The best response of the strain was recorded in presence of the fraction V (about 60 % of acetonitrile), since no interruption of vitality appeared. Actually the peak composition of the chromatogram in the fraction V area was the most different by passing from a flour to another (Figure 19).

Figure 19 RP-HPLC chromatograms of CEs in a C18 column eluted at a flow rate of 0.5 m min^{-1} with a linear gradient of solvent A (0.1% TFA in water) to 100% of solvent B (0.10% TFA in acetonitrile). Fractions from CE produced with flour of cv "Castagna di Montella" were collected, as indicated, every 10 min.



To conclude, analysis performed by RP-HPLC demonstrated that in the chestnut flour, obtained by cv "Castagna di Montella" there is one or more substances that specifically offer a marked resistance to simulated gastric juice, albeit characterized by a weak content. The elution of these substances with about 60% of acetonitrile indicated that they were characterized by a medium hydrophobicity, thus suggesting their amphipathic nature under a chemical and physical point of view. This property may lead to a specific adhesion to bacterial cell surface through the hydrophobic side (Schär-Zammaretti & Ubbink, 2003). On the other hand, the hydrophilic side, exposed towards the aqueous solution, may likely determine the detected resistance to simulated gastric juice. The cell immobilization contributed to the stability of all tested strainsin simulated bile juice, but, even in this case, was the addition of CEs to improve the viability of LAB strains.

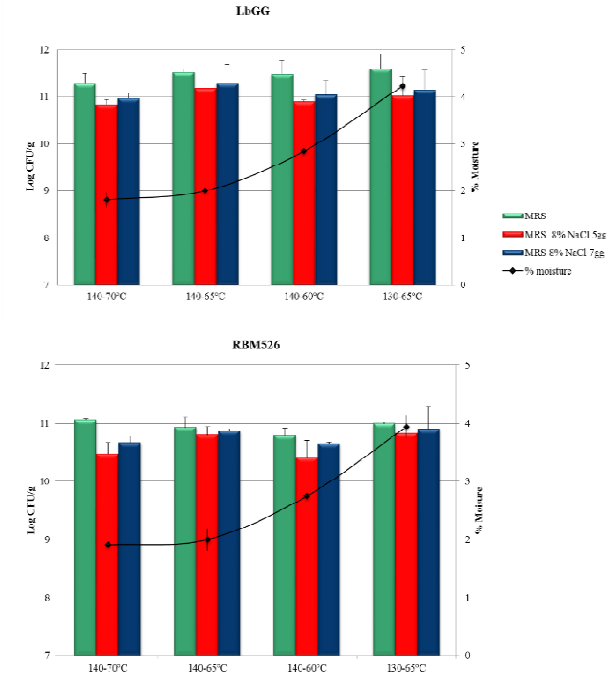
3.18.9 Thermal tolerance of probiotic strains

Two strains selected on the basis of their resistance to gastrointestinal transit, as well as on the ability to ferment chestnut purees were submitted to further test to define the resistance to heat treatments. According to heat-resistance tests, LGG and RBM526 strains did not significantly differ (p> 0.05) in their ability to survive at all treatment temperatures (data not shown). First of all, the maximum population level of both strains in CM appeared almost one log lower if compared with the growth

exhibited in MRS medium (data not shown). When strains were heat-treated at 60°C for 30 s, a reduction in the cell numbers, to about 3.70 log CFU mL^{-1}, was recorded in all cases. When temperature was increased to 70°C, a completely different trend could be observed: after 240 s, the population levels, if strains were grown in MRS and resuspended in CE, were still around 5 log CFU mL^{-1}, while dropped around 2 log CFU mL^{-1} for strains resuspended in trehalose solution, as well as in CE, if strains were grown in CM instead of MRS (Data not shown).
Undeniably, values for counts in trehalose of LbGG appeared in good agreement with those obtained by Sunny-Roberts and Knorr (2009) for the same strain. With temperature increasing (70°C), promising results were recorded when strains were grown in MRS and resuspended in CE. Actually, for preserving the function of micro-organisms in the dry state, sucrose and trehalose have been utilized the most (Ying et al., 2012), although other sugars (e.g. maltose and fructose) and sugar alcohols (e.g. sorbitol and inositol) may offer protective effects as well (Ying et al., 2012). As matter of fact, according to previous studies, CE, above all if produced with flour from the cv 'Castagna di Montella', is highly rich in reducing sugars and small peptides (Blaiotta et al., 2012, 2013) and, it is well documented that carbohydrates have protective effects for probiotic bacteria during drying (Corcoran et al., 2004). Surprisingly, no advantage was recorded when strains were cultivated in CM instead of MRS. Actually, the presence of protectants in the media, prior to fermentation, generally leads to an adaptation of probiotics to the environment, as compatible compounds accumulate within the cells, and the osmotic difference between the internal and external environments is reduced (Meng et al., 2008).

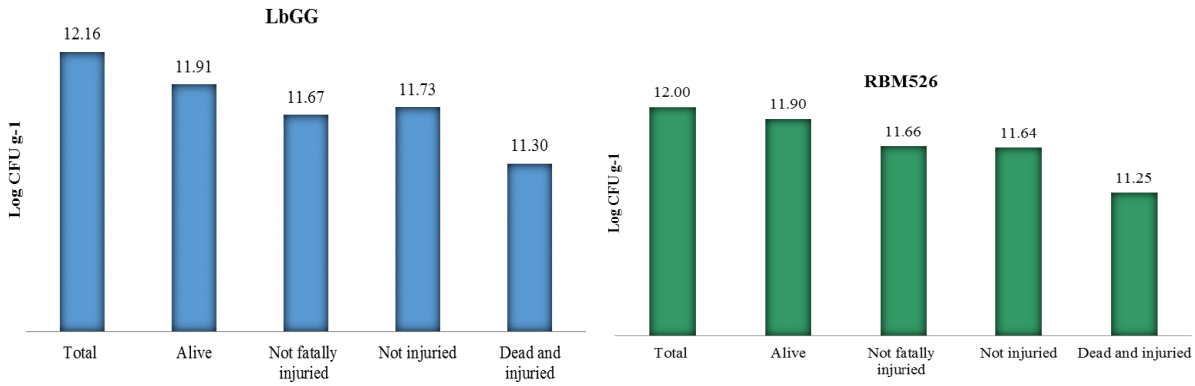
3.18.10 Survival rates during spray drying
Apart from the survival rate, the main concern about spray drying is still the integrity of the cell that may be compromised by the exposure to high temperatures (Silva et al., 2011). As a matter of fact, this process may affect a large number of cellular components, including DNA, RNA, cytoplasmic membrane and cell wall (Santivarangkna et al., 2008). Because some probiotic properties are closely related to the structure of the bacterial surface, in the light of the further applications of the strains, it was of primary relevance to evaluate the cellular injury after treatment and to quantify the loads of undamaged cells, rather than the sole survival rate. Absolute viable counts are rarely provided in manuscripts focused on drying of cultures (Corcoran et al., 2004; Ananta et al., 2005; Chávez & Ledeboer, 2007), but if the aim of the survey is, as in this case, the employment of the strains for the production of probiotic food, the number of viable bacteria must be properly quantified and has to fall within the range recommended to potentially obtain a health benefit in the finished product.
Preliminary spray-drying experiments were conducted to determine the optimal thermal levels for probiotic viability and to obtain powders with moisture contents of ~3.5%. According to data reported in Figure 20, for the high water content, coupled with a troublesome stickiness of the powder, the combination inlet/outlet temperature 135/65°C could not be further considered, in spite of the recorded high survival rates (56.53 and 5263% for LbGG and RBM526, respectively). In the other three combinations, the residual moisture content was seen to decrease as the inlet air temperature increased, from 2.84 to 1.81% and 2.75 to 1.91%, for LbGG and RBM526, respectively (wt wt^{-1}; Figure 20). Following spray drying, the probiotic strains became sensitive to NaCl, as revealed by decreases in cell numbers in the presence of salt. With inlet fixed at 140°C, the percentage of not fatally damaged cells, by counting on MRS NaCl-added after 5 days of incubation, appeared significantly higher ($p< 0.05$) at an outlet temperature of 65°C, so the combination 140/65°C was used for further assessments. In these conditions, the obtained powders presented a moisture content around 2.0%, well below 3.5%, namely the level regarded as a good quality parameter of dried products for prolonged storage and stability (Zayed & Roos, 2004).
As expected, following spray drying, the probiotic strains became sensitive to NaCl, as revealed by decreases in cell numbers in the presence of salt. In general terms, growth in media supplemented with NaCl showed that injured cells were able to resuscitate. In fact, differences in cell counts between media with and without NaCl appeared lessened after prolonged incubation, as already reported in a previous survey (Mauriello et al., 1999). This suggested that cell membranes were damaged by the drying process, thus resulting in lower survival (Teixeira et al., 1996). Obtained data were similar to those reported by Gardiner et al. (2000) and Sunny-Roberts and Knorr (2009) on spray-dried lactobacilli. Moreover, results fit well with the outcomes of Corcoran et al. (2004) on strain LbGG: although LbGG showed an humble thermal tolerance in challenge tests, it exhibited a good survival during spray drying, confirming that thermal tolerance alone is not an accurate predictor of performance during spray drying and that other phenomena, such as dehydration, affect cell viability during drying. Actually, dehydration inactivation was associated with cell damage of *Lb. plantarum*, rather than thermal inactivation, during drying (Lievense et al., 1994).

Figure 20 Viable counts and cell damage, indicated by sensitivity to NaCl (log CFU g^{-1}), of spray-dried cultures (LbGG and RBM526) in chestnut extract at different inlet and outlet air temperatures. The line indicates the moisture contents of resulting powders.



To get the most reliable quantification of the cells not injured by the heat exposure, a parallel approach was adopted. For cells spray-dried at a constant air inlet temperature of 140°C, outlet temperature of 65°C and flow rate of 4.5 mL min^{-1}, bacterial viability per gram of dried powders was evaluated. In detail, total cells were obtained by direct counting at the microscope, alive cells were quantified by counting on MRS agar plates, undamaged cells were obtained as green cells by Live/Dead BacLight Kit and by counting on MRS agar plated added of NaCl, while the sum of damaged and dead cells was obtained by Live/Dead BacLight Kit (red cells; Figure 21). Quantification of undamaged cells by MRS salt-added counting provided the same result of Live/Dead staining (Figure 21).

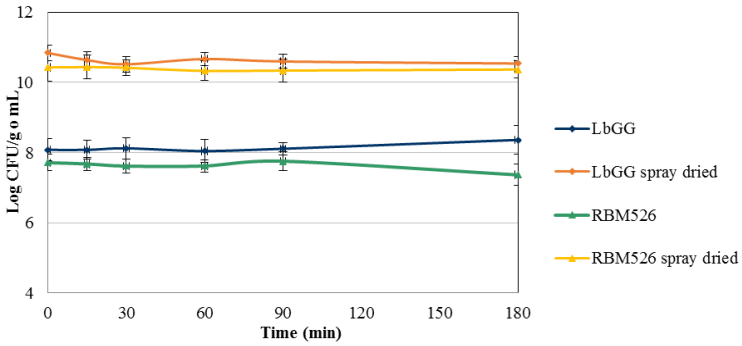
Figure 21 Viability (log CFU g^{-1}) of *Lb. rhamnosus* strains (LGG and RBM526) after spray-drying at an inlet temperature of 140°C, an outlet temperature of 65°C and a flow rate of 4.5 mL min^{-1}. Results are means on triplicate trials (±SD).



In other words, over 10^{11} cells g^{-1} (survival rate of about 50%), still presented a whole membrane. Data are in agreement with those reported by Corcoran et al. (2004) for cultures of LbGG in stationary phase of growth. Actually, the highest resistance of bacteria, if in stationary phase, has been associated with nutrient starvation (Santivarangkna et al., 2007) and to cross-protection conferred by the low pH attained during growth under uncontrolled pH conditions (Silva et al., 2005).

Both strains were able to survive in simulated gastric juice (Figure 22). The number of cells able to stand the acidic environment was the same obtained by undamaged cells quantification, namely around 11 log CFU g^{-1}. Probiotics endurance is dependent on the spray-drying media applied, as differences in thermal conductivity and diffusivity can affect survival of spray-dried probiotics (Lian et al., 2002). CE is an aqueous solution of reducing sugars, proteins, minerals and soluble carbohydrates with differing degrees of polymerization (Blaiotta et al., 2013).

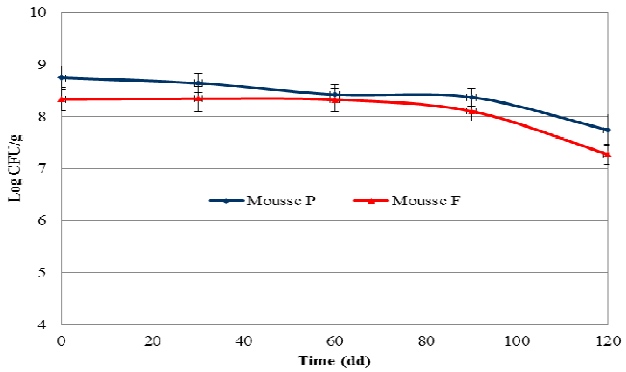
Figure 22 Viability of *Lb. rhamnosus* strains (LGG and RBM526) in presence of fresh chestnut extract (dashed lines) and as spray-dried cells in chestnut extract (continuous lines) during 180 min of incubation in presence of simulated gastric juice. Values are means of three determinations (±sd).



As already reviewed by Crowe et al. (1998), the use of sugar as protectants of dehydrated biomaterials such as enzymes, proteins, liposomes, red blood cells and bacteria can alternatively be explained by the water-replacement hypothesis, which envisages the function of sugars as water substitutes when the hydration shell of proteins and water molecules around polar residues in membrane phospholipids are removed. The replacement leads to maintenance of phospholipids bilayers at their hydrated spacing, which in turn preserves the structure of the membrane, thereby preventing damage during drying or freezing (Ananta et al., 2005). On the other hand, the ability of sugars to stabilize proteins during drying is due to the aptitude of the sugars to form hydrogen bonds with the proteins when water is removed, and to, hereby prevent protein denaturation by maintaining the protein structure (Crowe et al., 1998; Ananta et al., 2005).
Loads obtained in this study were about two log higher than those reported by Corcoran et al. (2004) and Sunny-Roberts and Knorr (2009) for LbGG (~10^9 CFU g^{-1}), but it has to be considered that a low ratio cells/carrier was used in the present study. In several studies, the influence of solids' concentration on the survival of micro-organisms during spray drying has been reported (Boza et al., 2004; Ananta et al., 2005). To conclude, data provided an indication of the suitability of CE as carrier and protector during spray drying and a confirmation of the ability of CE to allow probiotic bacteria to withstand the environmental stresses in the human GIT, even in a dried form.

3.18.11 Development of chestnut mousses enriched with functional lactobacilli
Spray-dried cultures of the two functional lactobacilli were incorporated in an anhydrous basis for chestnut mousse, stable at room temperature, developed *ad hoc*. Mousses have already been exploited as potential carriers for probiotic micro-organisms (Aragon-Alegro et al., 2007; Cardarelli et al., 2008; Buriti et al., 2010). In all cases, living bacteria were added to fresh mousses with the aim to develop a short shelf-life product, needing cold chain to be marked.Loads of *Lb. rhamnosus* LbGG and RBM526 in the anhydrous basis for chestnut mousse were initially 8.74 and 8.33 log CFU g^{-1}, respectively (Figure 23). A loss of viability was detected; nevertheless, both strains maintained a population level above 8 log CFU g^{-1}, during the first 90 days of monitoring (Figure 23). These amounts are comparable to those of milk-based probiotic products, for example bioyogurt, containing about 10^6 CFU of probiotic bacteria per ml at the end of their shelf life, which does not exceed 30 days when stored under refrigeration.

Figure 23 Viability of spray dried *Lb. rhamnosus* strains, (♦) LGG and (M) RBM526, in chestnut mousses during storage at 15°C. Data are the means of three replicate storage trials (±SD).



In point of fact, according to Boylston et al. (2004) and to Laurens-Hattingh and Viljoen (2001), the recommended level of probiotic micro-organisms in food at the time of consumption should be at least 10^6 CFU g^{-1} to exert beneficial effects on the consumer's health. According to this criterion, chestnut mousse may represent a potential vehicle for the tested strains. To evaluate the potential effect of lactobacilli and CE on sensorial profile of chestnut mousse, sensorial analyses of the three kinds of chestnut-based mousses (P, F and C) were carried out. Table 16 shows the mean scores of the sensory attributes selected by descriptive sensorial analysis to describe the samples and the overall acceptance of the experimental mousses. Only five descriptors (compactness, cocoa odour, taste intensity, sweet and texture) significantly varied ($p< 0.05$) within mousses samples (Table 16). With reference to samples aspect, only insignificant colour differences could be observed, while it was possible to discriminate C and F samples for compactness ($p< 0.05$). When focusing on odour and taste, all chestnut mousses were characterized by a cocoa flavour (4.5–6.6 mean intensity ratings) and taste intensity (6.4–7.3 mean intensity ratings). Lower but detectable levels of vanilla and chestnut flavours (<4.6 ratings) were perceived. Creamy and melting descriptors were not statistically different ($p< 0.05$), indicating that living supplements (strain LbGG or RBM526) did not exert negative effect. Similarly, no significant difference was observed for overall impression, exhibiting in all cases a good range of evaluation (> 6.5 ratings).

Table 16 Sensorial descriptors mean scores and standard deviations of chestnut mousses: control (C), probiotic mousse (P) and functional mousse (F).

	C	**P**	**F**
Aspect			
Brown colour	3.63^{a}±1.40	3.88^{a}±1.73	3.88^{a}±2.03
Compactness	7.25^{b}±0.89	$6.75^{a,b}$±1.67	6.00^{a}±0.93
Odour			
Intensity	6.88^{a}±1.13	6.25^{a}±1.28	6.50^{a}±0.93
Cocoa	6.63^{b}±1.69	5.50^{a}±0.93	6.38^{b}±1.41
Vanilla	4.63^{a}±1.92	3.50^{a}±2.07	3.88^{a}±2.42
Chestnut	4.25^{a}±2.05	4.16^{a}±1.46	4.25^{a}±1.04
Gustative			
Cocoa	6.38^{a}±1.19	6.13^{a}±0.83	6.13^{a}±1.46
Chestnut	4.50^{a}±2.45	5.00^{a}±2.00	5.00^{a}±2.20
Taste intensity	7.25^{b}±1.16	6.38^{a}±0.52	$6.88^{a,b}$±0.83
Sweet	6.38^{a}±1.41	7.25^{b}±1.04	6.00^{a}±1.51
Astringency	3.25^{a}±1.28	2.50^{a}±1.20	3.00^{a}±1.60
Bitter	2.75^{a}±1.39	3.38^{a}±1.60	3.75^{a}±1.49
Bitter aftertaste	2.63^{a}±1.19	2.38^{a}±0.74	3.00^{a}±1.41
Tactile			
Texture (soft/hard)	7.38^{b}±1.51	$6.63^{a,b}$±1.92	5.50^{a}±1.93
Creamy	5.75^{a}±2.25	6.63^{a}±1.19	5.38^{a}±2.39
Melting	6.88^{a}±1.13	6.13^{a}±1.89	5.88^{a}±1.46
Overall impression	7.38^{a}±1.51	6.88^{a}±0.35	7.13^{a}±1.13

Values with different letters in the same row indicate statistical differences according to the Duncan's test at $p<0.05$.

Actually, consumers' acceptance appeared to be related to creamy consistency as already highlighted by Elmore et al. (1999) for dairy desserts. Sensory results appeared in perfect agreement with those reported by Aragon-Alegro et al. (2007) for chocolate mousses or by Heenan et al. (2004) for frozen soy dessert supplemented with *Lb. acidophilus*: in all cases, probiotic variants could not be distinguished from the control sample. On the other hand, other authors reported that probiotic micro-organisms affected the flavour of the food product to which they were added. In the survey of Cardarelli et al. (2008), flavour of the probiotic and the probiotic inuline added chocolate mousses was significantly different, when compared to the control product after 14 days of storage but not at 7 days of storage.
Results here reported suggest that chestnut mousse, a food product naturally rich in antioxidant compounds, is an excellent carrier for delivering probiotic strains. Anhydrous powders for preparing ready to be prepared chestnut mousses represent a simple formulation of a functional food in which the probiotic strains, manufactured under industrial conditions, are able to survive and to retain their functionality during storage.

3.19 Conclusions

Results here presented indicate that chestnut fiber and above all chestnut extracts improve the tolerance of lactobacilli to simulated gastric and bile juice. Specifically, chestnut extracts exhibited a surprising effect in improving the tolerance to gastric transit of lactobacilli, while chestnut fiber mainly improved the tolerance to bile juice. These beneficial effects were put in relation with the peptides composition of the chestnuts extract and were proved to be, thus, dependent by the composition of the chestnut extracts and consequently by the cultivar used to produce flour. At the same time, results here reported suggest that chestnut mousse, a food product naturally rich in antioxidant compounds, is an excellent carrier for delivering probiotic strains. Probiotic lactobacilli have been directly incorporated as spray-dried cultures into the anhydrous basis and in this form the initial level of viable cells remained stable over 10^8 CFU g^{-1} during a three months long storage at 15°C. These amounts are comparable to those of milk-based probiotic products, e.g., bioyogurt, containing about 10^6 CFU of probiotic bacteria per mL at the end of their shelf life, which does not exceed 30 days when stored under refrigeration. The results obtained also suggest that anhydrous powders for preparing ready to be prepared chestnut mousses represent a simple formulation of a functional food in which the probiotic strains, manufactured under industrial conditions, are able to survive and to retain their functionality during storage. Moreover, according to sensorial analysis, all mousses exhibited a good acceptability and it was not possible to differentiate the control samples from the bacteria-added products. This is the first information on the survival of lactobacilli in an anhydrous basis for dessert: these initial assessments represent one of the possible applications of chestnut extract for probiotic strains protection along human GIT.

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3.21 Use of chestnut flour for the improvements of bakery goods

In order to develop gluten-free (GF) breads for celiac patients, a number of alternative flour types, such as corn, cassava, rice, soybean, chickpea and pseudocereals (e.g. buckwheat, amaranth and quinoa) have been evaluated to substitute wheat flour. Baking of GF flours is a challenge due to the lack of gluten proteins, as gluten is a protein which possesses structure-forming ability that affects elastic properties of dough and contributes to the overall appearance and crumb structure of many baked products. Therefore, the removal of gluten in GF formulation is a very demanding task resulting in often low quality, poor mouthfeel and low flavour products (Moroni et al., 2009).

In order to improve bread performance, sourdough could be used. The use of the sourdough process as a means of leavening is one of the oldest biotechnological processes in cereal food production. Sourdough is a mixture of mainly cereal flour and water, which is made metabolically active by a heterogeneous population of LAB and yeasts, either by spontaneous fermentation or by fermentation initiated through the addition of a sourdough starter culture, whether or not involving backslopping (De Vuyst et al., 2009). The partial abandonment of the traditional bread with "natural yeast", for the benefit of the industrial yeast, is mainly due to the possibility of simplifying the process, because the traditional system is generally more time-consuming and difficult to maintain stable over time. Currently, for the recognized and documented scientifically valuable characteristics of the bakery products sourdough-based, there is, worldwide, a renewed interest in this type of processing. Most rheological, sensory, nutritional advantages, and the recorded extended shelf-life, mainly derive from microbial metabolic activities, such as fermentation, proteolysis, synthesis of volatile compounds and antifungal activity (Hammes & Gänzle, 1998). The positive contribution of sourdough could be exploited for the production of high quality GF bread from various GF cereals and pseudocereals, with reduced need for expensive additives (Moroni et al., 2009).

In recent times, there has been a growing interest in the use of chestnut flour for the production of leavened bakery. Chestnut flour may be used in GF flour breads due to its nutritional and health benefits. Chestnut flour contains high quality proteins with essential amino acids (4-7%), relatively high amount of sugar (20-32%), starch (50-60%), dietary fiber (4-10%), and low amount of fat (2-4%). It also contains vitamin E, vitamin B group, potassium, phosphorous, and magnesium (Chenlo et al., 2007). Since most of the GF products do not contain sufficient amounts of vitamin B, iron, folate, and dietary fiber (Moroni et al., 2009), it may be advantageous to use chestnut flour due to its nutritional value (Demirkesen et al., 2010). Moreover, from a nutritional and prebiotic point of view, is very important that portion of the non-digestible starch, the so-called resistant starch (Moroni et al., 2009). Actually, studies on using of chestnut flour in bread making are gaining great interest in literature (Demirkesen et al., 2010). Obviously, the most important sensory characteristics that describe the flour and flour products are taste and aroma. The production of flavor compounds depends on the important variables, i.e., flour type and sourdough microbial flora. The generation of sufficient amounts of volatile compounds during fermentation needs a multiple step process of about 12-24 h, while fermentation by bakeries yeast alone is finished within a few hours (Hansen & Schieberle, 2005). As a matter of fact, sourdough bread has a higher content of volatiles and, also, achieves higher scores in sensory tests compared to, i.e. bread chemically acidified with lactic and acetic acid. Some of the compounds present in bread, i.e iso-butanol, iso-pentanols, ethyl acetate and ethyl lactate have been shown to be related to the concentrations in the corresponding sourdoughs (Hansen & Schieberle, 2005).

3.22 Materials and methods

3.22.1 Flours

Five flours (wheat, rice, buckwheat, maize, chestnut), kindly provided by Ipafood (Avellino, Italy), were used in the preparation of laboratory sourdoughs. Nutritional characteristics (%) can be reassumed as follows: chestnut flour - proteins 4.61, carbohydrates 69.3, fiber 9.50, fat 3.80, minerals 1.99; wheat flour - proteins 9.5, carbohydrates 73.5, fat 1.5, fiber 2.5, minerals 0.5, vitamins, 1.13 mg; buckwheat flour - proteins 11.5, carbohydrates 67.0, fat 2.6, fiber 2.6, minerals 1.6, vitamins 5 mg; rice flour - proteins 7.3, carbohydrates 87.8, fat 0.5, fiber 0.4, minerals 0.3, vitamins 4 mg; maize flour - proteins 8.2, carbohydrates 79.6, fat 1, fiber 3.1, minerals 2.4, vitamins 6.9 mg.

3.22.2 Laboratory mature sourdough preparation

Sourdoughs from mixtures of different flours were prepared according to procedure described by Hüttner et al. (2010) properly modified. The content of chestnut flour was kept constant at 40% (wt wt^{-1}). The remaining part of the mix was made up of wheat (A), buckwheat (B), rice (C), or maize flour (D). Furthermore, three sourdoughs were prepared with 100% of chestnut (E), wheat (F) or rice (G) flour. Sourdoughs were prepared from 100 g of flours and about 100 g of sterile water (yield dough about 200), in sterile 500 mL containers. Bakery yeast isolated in pure culture was added (about 10^7 CFU g^{-1}) at the sole first step. After 24 h of incubation, back-slopping was performed with 10% of the ripe sourdough and repeated every 24 h. Fermentations were propagated until a stable microbiota was established. Fermentations were carried in asepsis and at room temperature and each trial was performed in triplicate. At each refreshment step, reached volume (cm) was recorded and samples, taken from the ripe sourdough, were submitted to pH and total titratable acids (TTA) determination according to a standard method (Meroth et al., 2003). LAB and yeast loads were evaluated by counting on MRS modified according to Reale et al. (2011) and YGC (YG medium containing, per liter, 5 g of yeast extract and 20 g of glucose), respectively. For DNA

extraction and PCR-DGGE analysis, 10-g samples were taken from the sourdoughs (A to D) at seven selected times (24, 48, 72, 96, 120, 168 and 288 hours) and stored at -20°C.

3.22.3 Evolution of population dynamics in sourdoughs by PCR-DGGE

3.22.3.1 DNA extraction from sourdoughs

DNA was extracted from doughs (A to D) according to protocol developed by Iacumin et al. (2009).In detail, ten grams of sample were homogenized in a stomacher bag with 90 mL of saline/peptone water for 3 min. Fifty mL of supernatant were transferred into centrifuge tubes and subjected to a treatment at 80°C for 2 h, 100° C for 10 min and 0°C for 1 h. The mix was vortexed for 5 min and centrifuged at 6000 *g* for 10 min. The pellet was then re-suspended in 1 mL of TE buffer (10 mM Tris, 5 mM EDTA, pH 8). After a centrifugation for 10 min at maximum speed, the pellet was re-suspended in 120 mL of proteinase K buffer (50 mMTris-HCl,10 mM EDTA, pH 7.5, 0.5% wt vol^{-1}, SDS) and transferred into 1.5 mL screw cup tubes containing 0.3 g of glass beads with a diameter of 0.5 mm. Twenty µL of proteinase K (25 mg mL^{-1}, Sigma-Aldrich) and 20 mL of lysozyme (50 mg mL^{-1}, Sigma-Aldrich) were added and the mix was treated at 50°C for 1 h. Then, 150 mL breaking buffer (4% Triton X-100 vol vol^{-1}, 2% SDS wt vol^{-1}, 200 mM NaCl, 20 mM Tris pH 8, 2 mM EDTA pH 8) and 300 µL of phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.7, Sigma-Aldrich) were added. Three 30 s treatments at the maximum speed, with an interval of 10 s each, were performed in a bead beater (Fast Prep; Bio 101, Vista, CA). 300 µL of TE buffer (10 mM Tris, 5 mM EDTA, pH 8) were added and the tubes were centrifuged at 12,000 *g* at 4°C for 10 min. The aqueous phase was collected and precipitated with 1 mL ice-cold absolute ethanol. DNA was collected with a centrifugation at 14,000 *g* at 4°C for 10 min and the pellets were dried under vacuum at room temperature. DNA was finally eluted in 100 µL EB buffer.

3.22.3.2 PCR amplification of variable region V3 of 16S rDNA

In order to increase the amount of amplicons of hypervariable region V3 within 16S rDNA, nested PCRs were performed. In other words, the entire 16S rRNA was amplified by means of universal primers (FD1 and RD1) designed by Weisburg et al. (1990) and used as template for PCR amplification of hypervariable regions V3. Conditions, temperature profile and reaction mixture, for 16S rDNA amplifications were the same reported by Aponte et al. (2008). The primers V3f and V3r (Muyzer et al., 1993) were used to amplify the variable regions V3 of the 16S rRNA gene, giving PCR products of about 200 bp. To the forward primers, a GC clamp was added according to Muyzer et al. (1993). Each mixture (final volume, 25 µL) contained 1 µL of template DNA (16S rDNA amplification product), each primer at a concentration of 0.2 µM, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM MgCl2, 2.5 µL of 10X PCR buffer (Invitrogen) and 2.5 U of Taq polymerase (Invitrogen). A "touchdown" PCR was performed as previously described (Aponte et al., 2008) to increase the specificity of amplification and to reduce the formation of spurious by-products. All amplifications were performed in a programmable heating incubator (Techno, Progene, Italy). Aliquots (5 µL) of PCR products were routinely checked on 2% agarose gels.

3.22.3.3 Denaturing gradient gel electrophoresis (DGGE) analysis

PCR products were analysed by DGGE using a Bio-Rad D-code apparatus and the procedure first described by Muyzer et al. (1993). Samples were applied to a 8% (wt vol^{-1}) polyacrylamide gels in 1X TAE buffer. Parallel electrophoresis experiments were performed at 60°C by using gels containing 30-60% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40%, wt vol^{-1}, formamide) increasing in the direction of the electrophoresis. The gels were run for 10 min at 50 V and 4 h at 200 V, stained with ethidium bromide for 5 min and rinsed for 20 min in distilled water. Bands were visualized using a UV transilluminator (Gel Doc EQ, Bio-Rad).

3.22.3.4 Sequencing of DGGE bands

DGGE bands to be sequenced were purified in water according to Aponte et al. (2008). One µL of the eluted DNA of each DGGE band was re-amplified by using the above-described primers and conditions. PCR products which gave a single band co-migrating with the original band were then purified by QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions, and sequenced by Gene Chron (Roma, Italy). Research for DNA similarity was performed with the National Centre of Biotechnology Information GenBank.

3.22.3.5 Isolation, identification and molecular characterization of LAB cultures.

Forty-three LAB cultures were isolated from modified MRS (Reale et al., 2011) counting plates seeded with the highest dilutions. Isolates were purified by repetitive streaking onto MRS agar plates. DNAs, extracted according to Aponte et al. (2012), were used for strain biotyping by means of RAPD-PCR analysis with primer M13 (Huey & Hall, 1989), according to the protocol described by Aponte et al. (2012). Gels were analysed using the Bionumerics software, version 5.1 (Applied Maths, Kortrijk, Belgium). RAPD-PCR patterns were grouped by means of cluster analysis with the Pearson's product moment correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA). The profiles generated were concatenated by means of Bionumerics software to obtain a single dendrogram. The representative strains of the obtained

pattern groups were submitted to 16S rDNA gene PCR amplification (Weisburg et al., 1990) and further sequencing.

3.22.4. Characterization of volatile compounds (VOCs)

3.22.4.1 Head space solid phase microextraction (HS-SPME) analysis

The volatile fraction of samples was analysed by headspace sampling, using the solid phase microextraction technique (HS-SPME). For each SPME analysis, 2 g of sourdough taken at 24 and 288 h was placed in a 20 mL headspace vial, adding 2 mL of distilled water and 5 µL of 4-methyl-2 pentanol (internal standard, 100 mg/L standard solution). The vial was placed in a thermostatic block on a stirrer at 40°C and the fibre was inserted and maintained in the sample head space for 30 min, than it was removed and immediately inserted into the GC–MS injector for the desorption of compounds. For the analyses, a silica fibre, coated with 85 µm of Carboxen–Polymethylsiloxane (Carboxen/PDMS) according to Kaseleht et al. (2011) was used (Supelco, Bellefonte, PA, USA).

3.22.4.2 Gas chromatography–mass spectrometry (GC–MS) analysis

Volatile analysis was performed by an Agilent Technologies (Agilent Technologies, USA) 7890A gas-chromatograph coupled to an Agilent Technologies 5975 mass spectrometer equipped with a 30 m x 0.25 mm ID, film thickness 0.25 µm capillary column (HP-INNOWAX, Agilent Technologies, USA). Gas carrier was Helium (flow 1.5 mL/min) and SPME injections were splitless (straight glass line, 0.75mm I.D.) at 240°C for 20 min during which time thermal desorption of analytes from the fibre occurred. The oven parameters were as follows: initial temperature was 40°C held for 3 min, followed by an increase to 240°C at a rate of 5°C/min, then held for 10 min. Injector temperature was 240°C. Mass spectrometer operated in scan mode over mass range from 33 to 300 amu (2 s/scan) at an ionization potential of 70 eV. Mass spectral matches were made by comparison of mass spectra and retention time with those of MS database (Wiley7, Nist 05). A semi-quantitive analysis was obtained by comparison of the VOCs peak areas with that of internal standard (4-methyl-2 pentanol), obtained from the total ion chromatograms, using a response factor of 1. Blank experiments were conducted in two different modalities: blank of the fibre and blank of the empty vial. These types of control were carried out after every 20 analyses. All the analyses were performed in triplicate.

3.22.5 Statistical analysis

Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance was carryed on in order to evaluate the effect of fermentation on physico-chemical and microbial parameters as well on VOCs groups. Significant differences between the detected parameters were compared by means of Duncan's multiple comparison test at the 95% confidence level ($p \leq 0.05$). For each VOCs group and type of sourdough, statistical differences were evaluated for data at fermentation times by using a t-Student test where $p< 0.05$ was considered as statistical significant. Furthermore, a principal component analysis (PCA) was performed to visualize possible relationships within the data matrix. To decide the number of principal components (PCs), the eigenvalues of the correlation matrix, indicating the percentage of variability explained by each component, were tabulated and a scree plot was constructed.

3.22.6 Analysis of technological properties of LAB strains.

Urease activity was detected using a modified phenol red method (Mora et al., 2002). LAB strains were grown overnight in modified MRS broth containing 5 g urea L^{-1} and 0.05 g L^{-1} nickel sulphate. Bacterial cells harvested by centrifugation were washed twice with sterile 0.85% (w vol^{-1}) saline solution and resuspended in 100 µL of sterile saline. The assay mixture contained 100 µL of cell suspension and 400 µL of a solution consisting of 1 volume of reagent A (2 g of urea dissolved in 2 mL of ethanol and 4 mL of sterile deionised water) and 19 volumes of reagent B (1 g KH_2PO_4 L^{-1}, 1 g K_2HPO_4 L^{-1}, 5 g NaCl L^{-1} and 20 µg phenol red L^{-1}). The reaction mixture was incubated at 37°C for 2 h. The development of a red-violet colour indicated positive urease activity.

Screening for phytase activity was performed according to Anastasio et al.(2009) Strains were grown overnight at 30°C in modified Chalmers broth without neutral red and with 1% of sodium phytate (Sigma-Aldrich). The cells were harvested by centrifugation at 10.000 *g* for 10 min, washed and resuspended in sterile quarter strength Ringer's solution. 5 µL of the microbial suspensions was spotted on modified Chalmers agar plates without $CaCO_3$ and with 1% of hexacalcium phytate (Sigma-Aldrich). The plates were incubated aerobically at 30°C and examined after 5 days of incubation for clearing zones around the spots. To eliminate false positive results, caused by microbial acid production, cells were washed from the agar surface and plates were flooded with a 2% (w vol^{-1}) aqueous cobalt chloride solution. After 20 min of incubation at room temperature, the cobalt chloride solution was removed and the plates examined for persistent clear zones.

Exopolysaccharide (EPS) production was tested in APT medium (Difco) supplemented with filter-sterilized solutions of different carbohydrates (1% vol vol^{-1}) and ruthenium red (0.08 g L^{-1}). Glucose, maltose, starch, saccharose and fructose were added one by one.

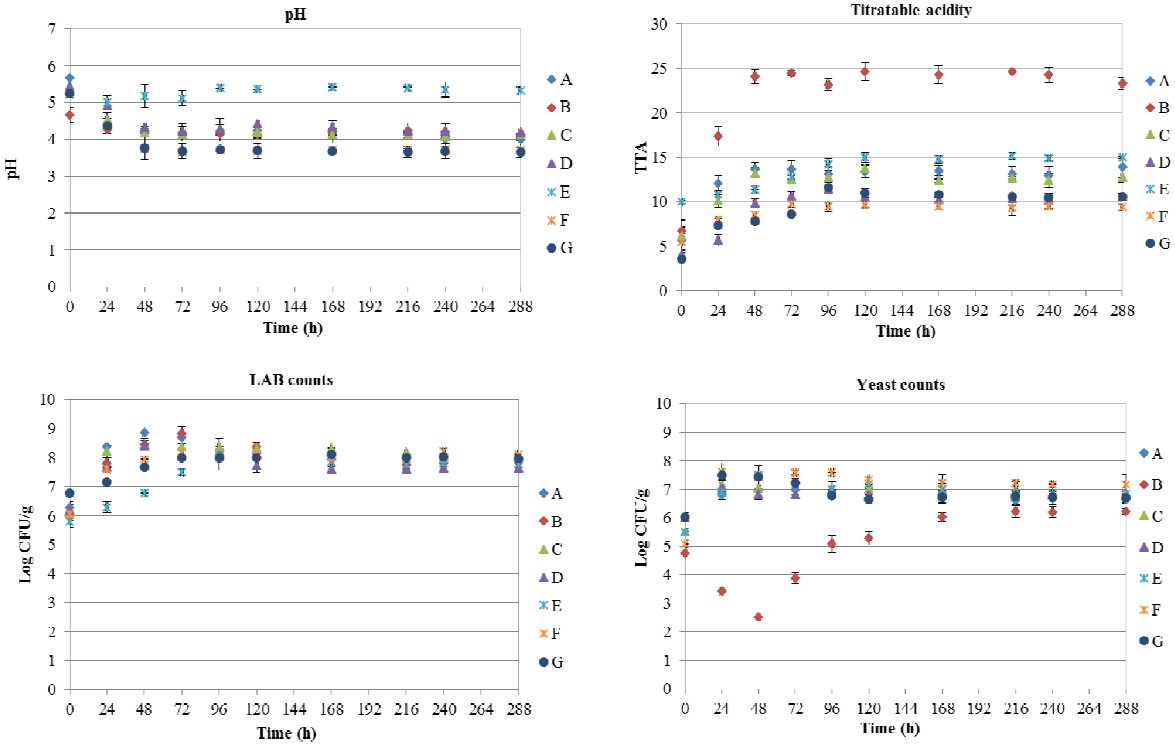
Acid fermentation by maltose, fructose, xylose, arabinose, and fructose plus maltose was evaluated by growth in MRS medium (pH 7.2-7.8) without glucose and Lab Lemco powder, but supplemented with phenol red (0.17 g L^{-1}) and filter sterilized sugar

solutions (0.5%).The ability to hydrolyze starch was evaluated by using starch agar plates drop-inoculated. After incubation at 30°C up to colonies visible growth, plates were covered with Lugol solution for one minute. After washing, strains were considered positive if a clear halo around colonies could be detected.
β-glucosidase activity was assayed using 4-nitrophenyl β-D glucopyranoside (Fluka, Milan, Italy) according to the method proposed by Fia et al. (2005).

3.22.7 Fermentative abilities of combinations of strains
Multiple combinations of strains were evaluated for the fermentative ability in terms of acids production and gas development according to Coda et al.(2010). Mixtures (20 g) of rice and chestnut flours (60:4) were mixed with cell suspensions (about 12.5 mL) of LAB grown for 24 h at 30°C in modified MRS broth. In detail, cells were collected by centrifugation (14.000 rpm, 10 min.), washed in sterile potassium phosphate buffer (50 mmol L^{-1}, pH 7.00) and resuspended in the same volume of sterile tap water. Controls were prepared by mixing flours blend with sterile phosphate buffer. LAB load, as well as volume increase, pH and TTA at time zero and after 24 h of incubation at 30°C were evaluatedaccording to a standard method.

3.23 Results and discussion
3.23.1 Sourdoughs characteristics
Volumes reached by samples throughout monitoring were, as expected, higher in gluten containing sourdoughs (A and F). Among GF flours combinations, promising results were obtained by combination of rice and chestnut flours: leavening was 2.5 cm after 24 hours and 1.3 cm at the end of monitoring (Data not shown). In all trials, the highest volumes were recorded during the first three days of refreshment (Data not shown). At the initial time of 24 hours, samples of chestnut flour blend (A-D) were all characterized by different pH values (range 4.66±0.20 - 5.67±0.01), but by the day two up to the end of monitoring values of sourdoughs obtained by mix of flours stayed almost stable and, at the end of monitoring (288 h), pH values ranged between 4.00 and 4.20 (Figure 24). The highest pH values ($p< 0.05$) were recorded for the sourdoughs entirely realized with chestnut flour (E). On the other hand, lower values ($p< 0.05$) were detected for sourdoughs made up of 100% wheat (F) or rice (G) flour (Figure 24). TTA exceeded the value of 10 at the end of fermentation for all sourdoughs except for trial F (9.40±0.35). Sourdoughs produced by mixing buckwheat and chestnut flours (B) exhibited the highest TTA levels (Figure 24). These values appeared greater than those reported by Moroni et al. (2010) for buckwheat flour, but congruent with those reported by Vogelmann et al. (2009). The higher TTA values of sourdoughs realized with chestnut flour (E) can be put in relation with the presence in chestnuts of several organic acids such as malic, oxalic, citric, ascorbic, quinic and fumaric acid (Ribeiro et al., 2007). At any extent, analyzed samples did not show a linear correlation between pH and TTA values.
By the day four a stabilization of LAB population was reached. Loads recorded on MRS medium ranged between 7.65±0.02 (D) and 8.12±0.03 (F) Log CFU g^{-1} at 288 h (Figure 24). The highest population level (about 8.80 Log $CFUg^{-1}$) was attained after only 48 h in trial A, while in trial B the almost same level of LAB population required further 24 h. On the other hand, the sourdoughs of sole rice (G) and, above all the ones based on 100% of chestnut flour (E) required a longer time (96 h) to achieve the equilibrium (Figure 24). Except for trial B, based on mixing of buckwheat and chestnut flours, a stable yeast microbiota was achieved by the day four with loads, at 288 h, ranging between 6.23±0.09 (B) and 7.16±0.35 (F) Log CFU g^{-1} (Figure 24). An unusual trend was recorded in trial B: after an initial drop, yeast loads stabilized around 6 Log CFU/g by the day five. Indeed, inhibition of yeasts growth was described in buckwheat sourdoughs developed by spontaneous fermentation or addition of starter cultures (Vogelmann et al., 2009; Moroni et al., 2010). Yeast/LAB ratio was around 1:10 in almost all the samples, reflecting the typical proportion existing in mature sourdoughs (Reale et al., 2011).

Figure 24 Evolution of LAB, yeasts and measured chemical parameters at each refreshment step during the course of propagation of sourdough fermentation batches A-G.



3.23.2 Monitoring of the sourdough microflora

To investigate fluctuations within the bacterial population and to trace back the origin of LAB species, samples of sourdoughs obtained by mixing chestnut flour with wheat, buckwheat, rice or maize flours (A-D) were firstly evaluated by a culture independent approach PCR-DGGE based. Samples were collected at 24, 48, 72, 96, 120, 168 and 288 h and submitted to DNA extraction followed by DGGE analysis of V3 amplicons. Major bands obtained were excised, checked for co-migration, sequenced and submitted to BLAST comparison with the nucleotide database of the National Center of Biological Information (NCBI). In all cases, the culture independent approach evidenced an extremely low bacterial variety. Dominant species could be related to the genera *Lactobacillus* spp and *Pediococcus* spp (Table 17). In detail, a band, common to all sourdoughs could be reported, with a similarity level of 99% at least, with the following *Lactobacillus* species: *Lb. fabifermentans* (GenBank: accession no. NR042676.1), *Lb. plantarum* (NR042394.1), *Lb. plantarum* subsp *argentoratensis* (NR042254.1), *Lb. paraplantarum* (NR042676.1) and *Lb. pentosus* (NR029133.1). These species, referable to the so-called *Lb. plantarum* group, have a 16S rRNA sequence similarity of over 99% and may be distinguish by hsp60 sequencing or hsp60 PCR-RFLP analysis (Blaiotta et al., 2008). Actually, members of the *Lb. plantarum* group are considered the main species dominating sourdough fermentation processes that are characterized by low incubation temperatures and continuous backslopping (De Vuyst et al., 2009). *Pediococcus* (*Pd*) *pentosaceus* was the main species retrieved among pediococci; even in this case, exponent of this taxon were visible as a major band at all the analysed times (Table 17). In all sourdoughs, except the trial with wheat flour (A), species belonging to the genus *Weissella* (*W.*) spp. were identified (Table 17). In trial B, namely sourdoughs produced by mixing buckwheat and chestnut flours, the occurrence of one band referable at 98% of similarity with *W.confusa* and *W.cibaria* does not surprise: *W. confusa* has already been detected in rye sourdoughs (Meroth et al. 2003), while *W. Cibaria* was reported as recurrent in oat (Hüttner et al., 2010), teff and buckwheat sourdoughs (Moroni et al. 2010). Likely, the competitiveness of *W. cibaria* during buckwheat/chestnut fermentations is probably due to its preference for glucose over maltose (Moroni et al., 2010). Obtained data resulted quite different from those reported in previous studies focused on the analysis of sourdoughs realized with the sole wheat, buckwheat, rice or maize flours. In sourdough rice, *Lb. curvatus*, indicated, by DGGE analysis, as the dominant species (Meroth et al., 2004), was not retrieved in the present study. *Lb. farciminis*, a typical sourdough LAB (Meroth et al. 2003; De Vuyst et al., 2009) was detected in wheat/chestnut (A) and rice/chestnut (C) sourdoughs, but not in those containing maize (D) or buckwheat flour (B) (Table 17).

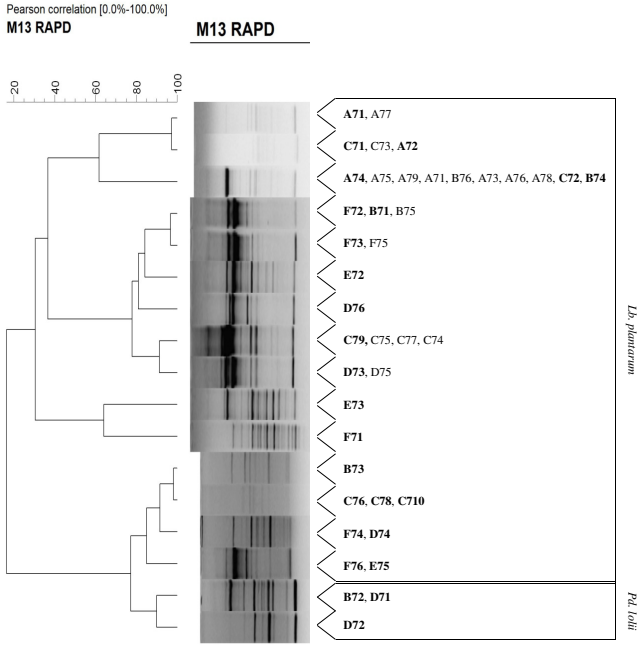
Table 17 Identifications obtained by blast comparison in Genebank of V3 16S rDNA region sequences after PCR-DGGE analysis. DNA directly extracted from samples collected during sourdoughs ripening. Sequences are reported following the order of migration on DGGE patterns.

Hours Sample	24	48	72	96	120	168	288	% of similarity
A						*Lb. plantarum* group	*Lb. plantarum* group	100
			Lb. plantarum group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	100
	Pd. pentosaceus	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*		100
							Lb. farciminis	100
	Desulfotomaculum spp.	*Desulfotomaculum* spp.	*Desulfotomaculum* spp.	*Desulfotomaculum* spp.	*Desulfotomaculum* spp.	*Desulfotomaculum* spp.	*Desulfotomaculum* spp.	89
B	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	100
			W. cibaria/confusa	*W. cibaria/confusa*	*W. cibaria/confusa*	*W. cibaria/confusa*	*W. cibaria/confusa*	98
	Pd. pentosaceus	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	100
	Pd. pentosaceus	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	100
	Pd. lolii/stilesii	*Pd. lolii/stilesii*	*Pd. lolii/stilesii*	*Pd. lolii/stilesii*	*Pd. lolii/stilesii*	*Pd. lolii/stilesii*	*Pd. lolii/stilesii*	100
C	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	100
	W. paramesenteroides	*W. paramesenteroides*					*Lb. farciminis*	100
	Pediococcus spp	*Pediococcus* spp		*Pediococcus* spp	*Pediococcus* spp	*Pediococcus* spp		96
D	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	*Lb. plantarum* group	99
				W. paramesenteroides	*W. paramesenteroides*	*W. paramesenteroides*		99
	Pd. pentosaceus	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	*Pd. pentosaceus*	100

A: Chestnut and wheat flour; B:Chestnut and buckwheat flour; C:Chestnut and rice flour; D:Chestnut and maize flour

Forty-one strains were isolated from mature sourdoughs (A to D) and genotipically characterized by means of RAPD PCR. One or more representative of each RAPD-type were identified by 16S rDNA partial sequence followed by BLAST comparison of the obtained sequences with the nucleotide database of the NCBI. Except three strains, isolated from buckwheat/chestnut (B) and maize/chestnut (D) sourdoughs, that could be identified as *Pd. lolii*, all cultures could be classified as belonging to the *Lb. plantarum* group, with percent of similarity of 99% at least (Figure 25). These results are in agreement with other studies on the bacterial population in Italian sourdoughs (Ricciardi et al., 2005; Catzeddu et al., 2006; Iacumin et al., 2009; Reale et al. 2011). *Lb. sanfranciscensis*, described as the dominant bacterial species in sourdoughs (De Vuyst et al., 2009) was not found in our samples, even if modified MRS medium (Reale et al., 2011), a selective substrate to isolate this species, was used. At any extent, RAPD-PCR analysis represented a suitable tool to evidence genotypic differences among strains within the same species. Except for strains isolated from trial A (sourdoughs from wheat and chestnut flours), that were, with three exception, all characterized by the same DNA fingerprint, RAPD-PCR highlighted a strong biodiversity among strains belonging to the same species and isolated from the same sourdough (Figure 25). This evidence should be considered as a feature which positively influences the characteristics of natural GF sourdoughs.

Figure 25 Dendrogram obtained from M13 RAPD-PCR fingerprints of 43 LAB strains isolated from mature sourdoughs. Patterns were grouped with the unweighted pair group algorithm with arithmetic averages (UPGM). Sequenced strains are in bold.



In this study, culture-dependent and culture-independent methods were combined to obtain a complete picture of the biodiversity of the different sourdough ecosystems. Considering the direct analysis of nucleic acids using the universal primers for bacteria an higher variability was recorded with members of *Lb. plantarum* group and pediococci equally largely represented in all trials, independently from the time of propagation. Conversely, the identification of over forthy strains allowed to identify, with three exceptions, *Lb. plantarum* strains. In other words, the bacterial ecology of the four samples obtained from direct analysis appeared different to that recorded by traditional plating followed by molecular identification of the isolates. The circumstance that isolation on the medium traditionally employed for sourdough analysis did not reflected speciography recorded by DGGE, could be related to the poor selectivity of the medium or to the strains isolation from the higher dilution plates, as already been reported (Iacumin et al., 2009), as well as to the possibility that these populations are present in viable non-culturable (VNC) state, due to the carbohydrate starvation.

3.23.3 Identification and quantification of aroma compounds

Although the main part of the aroma is produced upon cooking, many aroma precursors are formed during the fermentation process. The volatile compounds of sourdough bread are produced by biological and biochemical actions during fermentation and contribute to flavour (Hansen & Schieberle, 2005). To describe VOCs profiles of chestnut flour based sourdoughs, samples were analysed at 24 h and 288 h by means of SPME-GC/MS analysis. To our knowledge, only one study has reported the characterization of the volatile fraction of chestnut flour using HS-SPME approach (Cirlini et al., 2012).

From the volatile analysis of samples (A-E), 59 VOCs were found in sourdoughs obtained by 100% chestnut flour (E) and chestnut flour blends (A-D) at two fermentation times (24 h and 288 h). Table 18 lists all identified VOCs, split into many chemical classes: aldehydes (8), alcohols (16), esters (13), ketones (6), acids (2), furans (1), pyrazines (4), phenols (4), terpenes (2), lactones (1) and alkanes (2). VOCs of sourdoughs may derive from microbial metabolism (e.g. alcohols, 2,3-butanedione, 3-hydroxy-2-butanone, esters and acids), enzymatic oxidation or autoxidation of flour lipids (e.g. aldehydes, ketones and 2-pentylfuran), caramelization process under drying conditions and rearrangement of carbohydrates via Maillard reaction (e.g. furans, pyrazines, phenolic compuonds) and genetic and environmental factors (e.g. terpenes and lactones) (Rehmanet al., 2006). The most abundant alcohol among VOCs was 3-metil 1 butanol (isoamylalcohol), reported as the most important flavor active compound produced by yeast fermentation. Ethanol, 2-methyl-1-propanol, 1-propanol, 2-phenylethyilalcohol and 1-butanol were also found. The development of alcohols can be attributed to the activities of high levels of LAB occurring during steeping, as well as to the yeasts activity (Rehman et al., 2006). 2-furfurylalcohol could be ascribed to the caramelization process under drying conditions, which may involve free sugars naturally occurring in fresh chestnut fruits (Cirlini et al., 2012).

The appreciable quantities of aldehydes recorded may be due to lipoxygenase reactions initiated by ruptured vegetal cells during milling into flour (Peppard & Halsey, 1981). In particular, in the case of chestnut flour, all the aldehydes presenting a linear chain, such as hexanal and acetaldehyde, could result from the degradative oxidation of unsaturated fatty acids, especially oleic, linoleic and linolenic acids, in which chestnuts are rich (Pires Borges et al., 2007). The moderate concentration of hexanal in B and C, was likely to be related to the relatively high susceptibility to rancidity, for oxidative lipid breakdown, of rice and buckwheat flours (Grosch, 1982). Among aldehydes, 3-methyl butanal, likely produced by Strecker degradation of amino acids, was present in trial D and C, especially at 24 h. In fact, this compound is a common aroma already recorded in corn and corn products (Buttery & Ling, 1998) and was even retrieved in rice cakes (Buttery et al., 1999). Nonanal, the most abundant aldehyde found, could be a by-product from the decomposition of ethyl oleate (Peppard & Halsey, 1981). Aromatic aldehydes, such as benzaldehyde, were also found in all sourdoughs at 24 h and especially in trials A (200.7±5.7 ppb) and B (103.6±9.7 ppb), probably as result of aromatic amino acids degradation, while furfural could be attributed to the caramelization process under drying conditions. However, the origin of most other aliphatic aldehydes and alcohols, as well as of aliphatic hydrocarbons and 2-pentylfuran, is the oxidative and/or thermal degradation of unsaturated lipids (Grosch, 1982).

The most abundant ester was ethylacetate. Esters such as ethylpropionate, 3-methylbutyl acetate, ethylhexanoate, ethylheptanoate ethyloctanoate have also been reported in spontaneously fermented maize dough (Annan et al., 2003) and buckwheat whole grains and ground (Prosen et al., 2010). Ethyl lactate was found in all the sourdoughs (A-E) and its levels always increased with fermentation time.

Ketones were widely represented in trials E, B and D. Especially, 2,3-butadione and acetoin were detected in all sourdoughs at 24 h. Such compounds are important fermentation indexes. Beside aldehydes and ketones, γ-butyrolactone (2.5%), already reported as a compound characteristic of chestnut fruit and flour (Cirlini et al., 2012), was found in all samples. Alkanes were present only in wheat/chestnut flour sourdough (A). As expected, the level of acetic acid, the most abundant volatile acid produced during sourdough microbial fermentation by heterofermentative LAB, increased in all trials, except E (Table 18), in agreement with the recorded pH trend (Figure 24). Sourdoughs produced by buckwheat/chestnut flours blend (B) exhibited the highest values (2317±11.9 ppb after 24 h and 3202.8±12.3 ppb after 288 h), in agreement with reported TTA results (Figure 24).

Among phenols, 2-methoxyphenol (guaiacol), as well as p- and m-cresol, were found in all sourdoughs (A-E). These compounds, formed during the thermal degradation of lignin under pyrolysis conditions, could be ascribed to the smoke generated by burning chestnut wood or by thermal degradation of barks (Gruber, 2010).

Pyrazines were detected in low amount in A, B, C and D samples, but not in sourdough E. The production of these compounds has a striking effect on the aroma of wheat bread crust (Schieberle & Grosch, 1994), corn tortilla chips (Buttery& Ling, 1998), rice cakes (Buttery et al., 1999) and the buckwheat grains (Janeš et al., 2009). Limonene was lost during the fermentation process, while 1-borneol was found in B, as already been reported by Janeš et al. (2009).

In order to better characterize the aroma profile of sourdoughs and to study the effect of the fermentation on the VOCs, collected data were grouped (Figure 26). All sourdoughs were largely (98% of the total amount) dominated by alcohols (12.2-82.5%, B and A, respectively), esters (13.7-71.2%, A and B, respectively), acids (0.3-9.6%, D and B, respectively), aldehydes (1-9%, B and D, respectively) and ketones (0.1-4.2%, C and B, respectively). VOCs classes appeared affected by flour type and fermentation time. Alcohols in buckwheat/chestnut flours (B) and maize/chestnut flours (D) significantly ($p< 0.05$) increased after 288 h (Figure 26), but the amount was significantly ($p< 0.05$) lower than A, C and E.

Apart from ketones, the influence of fermentation time on major VOCs groups (esters, acids, aldehydes and terpenes) exhibited

a similar trend. In particular, the reduction of total aldehydes in D resulted higher (p<0.05) than in C, because the amount of 3-methylbutanal decreased its level by tenfold while linear aldehydes, such as hexanal and acetaldehyde, increased for lipid autoxidation. Instead, ketones group increased in A, B and C, while significantly (p< 0.05) decreased in D and E.
The results of the t Student test did not highlighted the existence of significant quantitative differences (p< 0.05) between the level of phenols and lactone for A, lactone for D and phenols for B. Alkanes were present only in wheat/chestnut flours sourdough (A) and decreased significantly (p< 0.05) during fermentation (Figure 26).

Figure 26 Changes in the fermentation compounds found in chestnut flour based sourdoughs (A-E) after 24 h (□) and 288 h (■) of fermentation. Within the VOCs group, means with different letters for the same fermentation time (24 or 288 h) are significantly different (p< 0.05). For each VOCs group and type of sourdough (A-E), significant differences (t-Student) were shown at 24 h (*: p< 0.05) and 288 h.

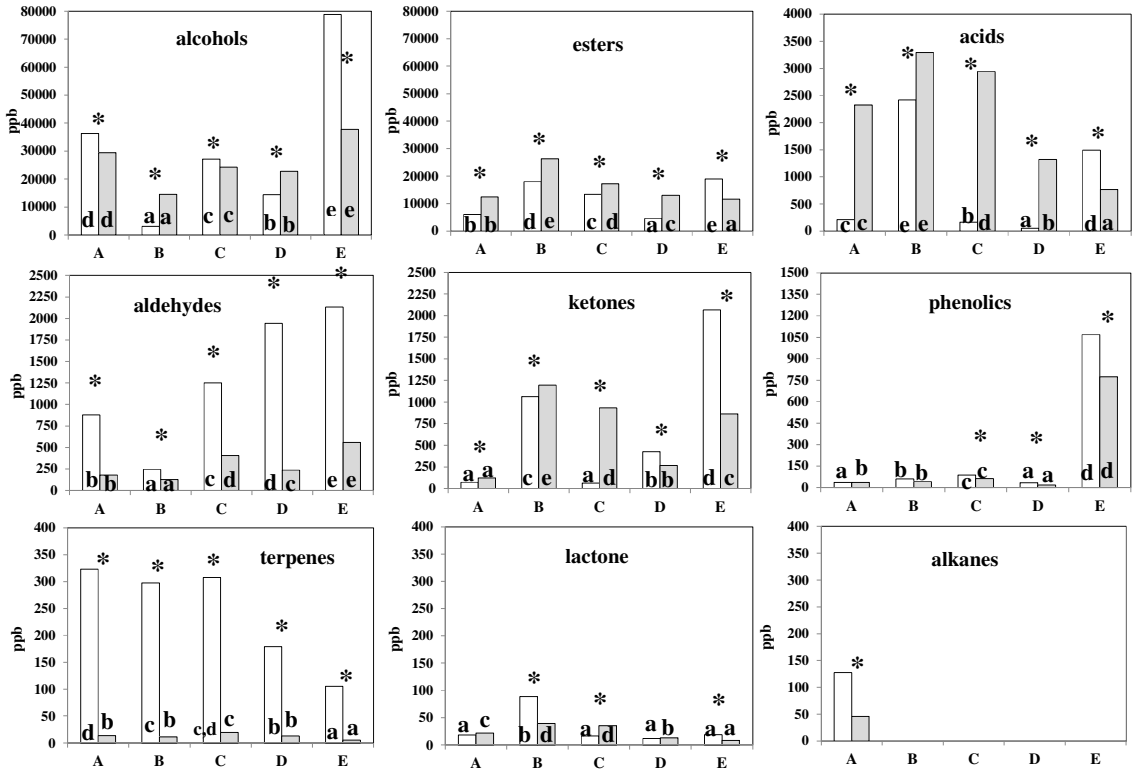


Table 18 Compounds detected in sourdoughs (A-E) after 24 h and 288 h of fermentation.Each value is expressed as Mean ± SD (ppb).

Compound	A (WF/CF)		B (BF/CF)		C (RF/CF)		D (MF/CF)		E (100% CF)	
	24h	288h	24h	288h	24h	288h	24h	288h	24h	288h
Acetaldehyde	125.02±1.7	144.72±9.8	51.14±0.1	42±5.6	54.21±2.7	218.78±9	126.27±12.6	91.21±8.8	456.87±9.8	491.55±3.6
2 methyl –propanal	7.26±0.8	9.1±2	nd	nd	37.7±2.8	nd	73.54±5.2	10.44±1	98.83±9.4	37.81±1.4
3 methyl –butanal	nd	nd	nd	nd	695.2±8.9	12.99±0.5	1153.8±10.3	101±4.3	nd	nd
Hexanal	nd	nd	72.9±1.6	38.01±9.1	82.4±5.3	142±1.3	20.2±1.4	22.7± 2.2	30.1±0.9	10±0.5
Heptanal	27.03±6.5	10±1.5	nd	nd	nd	nd	nd	nd	nd	nd
Nonanal	500.3±9.3	nd	16±1.1	nd	313.01±6.1	1.95± 0.9	475.9±9.5	nd	1268.2±14.1	nd
Furfural	17.53±0.6	nd	nd	nd	22.55±3.53	nd	13.27±0.9	4.62±0.4	37.61±3.6	20.31±0.7
benzaldehyde	200.7±5.7	16.31±1.9	103.6±9.7	46.01±5.7	47.18±1.4	29.48±3.1	48.22±3.4	6.40±0.6	39.22±3.7	nd
Ethanol	15700±133.9	12001.4±50	2000.4±24.3	9766.8±24.7	10800.1±26.1	8934.4±14.8	6955.4±64.1	11540.1±61.6	31890±142.7	11945.8±56.1
1-propanol	188.1±13.2	50.4±9.1	420.4±14.4	217±4.9	424.9±11.2	200.0±11.3	221.5±1.3	185.9±5.1	777.2±6	718.6±5.1
3-hexanol	nd	nd	3.30±0.22	nd	nd	nd	nd	nd		
1-butanol	67.4±3.2	107.9±14.6	10.8±0.7	13.6±0.4	92±8.9	113.6±8.3	36.7±2.6	55.1±5.3	321.4±6.6	149.4±5.3
1-penten-3-ol	23.57±10.1	nd	6.83±0.5	nd	nd	nd	154.25±3.2	273.01±3	nd	nd
isobutylalcohol	138.67±6.5	nd	62.78±4.3	nd	2068.6±8.8	1141.17±4.5	nd	nd	5757.17±15.1	673.48±3.5
isoamylalcohol	19177±31.5	16364.4±38.7	327.3± 22.2	4200.5±7	12676.8±53	12088.0±40	6720.7±13.7	10314.2±111	38828.6±34.7	22793.5±9.6
1-pentanol	113.13±6.98	136.26±2.9	nd	nd	209.49±10.8	297.45±2.1	63.16±4.5	51.17±4.9	366.54±5.6	254.11±1.5
2-penten-1-ol	6.36±7.3	0.44±0.1	nd	nd	nd	nd	nd	nd	nd	nd
3-methyl 2-buten-1ol	101.16±8.5	nd	nd	nd	nd	nd	nd	nd	nd	nd
cis 2-pentenol	nd	nd	17.76±1.2	25.21±0.8	nd	nd	nd	nd	nd	nd
1-hexanol	234.19±6.5	357.13±9.4	110.47±7.5	250.83±6.3	510.74± 0.6	1047.43±19.6	93.73±5.3	121.1±11.7	38.24±3.6	109.1±3.9
1-octanol	10.05±1.3	12.04±1.5	nd	nd	nd	43.5±1	nd	nd	nd	nd
2-furfuryl alcohol	16.56±2.9	15.95±2.9	72.91±4.95	nd	22.97±3.7	27.65±1.7	13.77±0.98	13.02±1.25	57.21±5.5	60.88±2.2
2-phenylethyilalcohol	522.78±14.9	388.24±2.9	52.27±3.5	77.38±20.5	345.45±7.9	337.29±8	132.93±3.1	171.73±16.5	356.42±4.6	1050.46±3.6
3-ethoxy 1-propanol	25.94±1.2	nd	nd	nd	44.1±3.6	20.47±1.9	17.54±1.3	7.41± 0.1	35.44±3.4	77.83±2.8
ethylacetate	4320.44±14	9489.42±27.9	16626.5±317	23349.4±267	9635.6±26.4	12656.9±125	3814.13±56.1	11401.6±98	14105.9±10.3	10490.4±37.2
methylacetate	15.6±0.65	75.52±1.3	736.43±50	27±5.8	21.43±0.4	44.45±3.3	nd	49.34±4.7	nd	nd
propyl acetate	700.01±11.2	1537.87±9.4	11.25±0.7	55.82±0.9	432.9± 2.1	194.48± 0.7	23.95±1.7	62.89±6.1	116.60±11.1	nd
2-methyl propylester[a]	17.42±3.2	145.81± 7.8	43.93±2.9	77.51±1.2	113.9±13.1	290.89±3.1	131.37±0.7	160.47±13.2	193.27±13.1	35.76±1.3
ethyl ester butanoic ac.	nd	nd	143.33±9.7	106.64±4.83	77.4± 3.8	62.42±2.1	nd	nd	243.38±23.2	nd
isoamyl acetate[b]	22.98±4.5	314.9±3.4	28.15±1.9	821.14±1.7	253.7± 12.9	1877.06±21.5	86.63±6.2	735.36±10	1601.32±3	409.69±1.3
ethyl pentanoate	87.07±3.3	16.88±1.6	67.84±4.4	99.62±1.6	225.4±7.4	51.07±2.1	75.06±5.3	26.88±2.6	nd	nd
ethyl hexanoate	595.18±4.5	357.17±7.8	43.95±2.9	19.9±0.89	2236.7±26.9	1529.4±8.5	361.9±11.42	282.7±4.23	700.37±13.4	368.12±7.4
ethyl lactate	149.33±8.6	415.36±13.7	242±16.4	1718.9±36.4	175.03±3.8	349.63±5.3	13.50±0.96	210.9±2.69	110.1±6.5	189.1±3.1
ethyl heptanoate	25.96±3.1	22.47±2.7	14.42±0.9	24.97±1.5	40.75±1.7	55.19±3.5	16.80±1.2	15.68±1.5	19.38±1.3	27.05±0.9
ethyl octanoate	57.93±8	38.36±0.5	9.01±0.6	12.98±0.2	69.75± 4.8	53.83±3.2	29.14±2	23.08±2.2	443.86±11.06	101.77±3.6
2-methyl ethyl ester[c]	7.46±0.6	nd	nd	nd	31.41± 2.8	35.97± 2.6	nd	nd	770.05±6.6	nd
2-furan carboxilic acid[d]	5.97±0.9	nd	nd	nd	8.28± 0.35	nd	45.43±3.24	nd	639.68±7.6	52.41±1.9
2,6-dimethyl octane	74.79±4.8	45.85±4.3	nd	nd	nd	nd	nd	nd	nd	nd
2,6-dimethyl undecane	52.58±7.2	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-3 butanedione	44.46± 5.8	51.34±4.8	819.59±41.21	159.39±2.6	36.37±1.7	99.11±1.07	250.45±18.3	126.61±12.19	337.96±5.5	nd
2-pentanone	nd	nd	nd	nd	nd	nd	108.33±0.19	nd	55.85±5.3	nd
2-heptanone	6.75±1.3	nd	4.55±0.31	nd	nd	nd	nd	nd	16.15±0.6	nd
acetoin	20.61±6.3	71.74±6.83	239.25±16.2	1036.10±31.4	26.97±2.2	834.29±8	66.97±4.8	140.36±2	1406.62±0.7	841.58± 11.1
2,3 pentanedione	nd	nd	nd	nd	nd	nd	nd	nd	231.47±22	nd
2methylciclopentanone	nd	nd	nd	nd	nd	nd	nd	nd	18.29±0.7	23.34±0.8
acetic acid	64.1±5.7	1911.3±4.3	2317±11.9	3202.8±12.3	165.5±8.9	2941.8±41.2	56.97±4	1309.4±64	1006.2± 10.8	721±5.1
2-methylpropanoic acid	149.3±4.6	415.4±13.7	80.29±5.6	90±1.2	nd	nd	nd	9.51±0.9	486.4±7	47.8±0.7
guaiacol[e]	5.02±0.3	10.4±1.9	26.3±1.8	25.2±3.3	29.7±0.3	33.1±6.9	9.4±0.6	4.4±0.4	985.9±12.7	194.1±6.9

phenol	28±3.5	24.1±2.1	33.38±2.2	16.5±9.7	50.1±4.7	21.4±2.0	21.5±1.5	11.4±1.1	46.7±4.4	481.1±3.4
m-cresol[f]	2.18±0.3	0.17± 0	nd	nd	8.09±0.1	6.91±0.3	3±0.2	0.95±0.1	17.54±1.6	57.40±2
p-cresol[g]	nd	nd	nd	nd	nd	nd	nd	nd	18.1±1.2	42.1±1.5
2-pentyl furan	3.9±0.2	6.1±1	10.5±0.7	6.1±0.4	1.9±0.1	5.8±0.7	1.9±0.1	7.2±0.7	5.4±0.7	7.8±0.1
limonene	322.6±12.4	13.2±0.8	297.2±10.2	11.2±0.3	307.3±9.6	19.6±3.7	179.0±5.6	12.7±1.2	105.1±5.9	5.0±0.2
1-borneol	nd	nd	43.18±2.9	33.99±2.9	nd	nd	nd	nd	nd	nd
γ-butyrolactone	18.0±1.5	21.7±1.9	88.4±6	39.1±3	16.5±4.1	35.2±3.2	12±0.8	12.9±1.2	18.4±0.1	8.2±0.2
2-methyl pyrazine	nd	nd	nd	nd	3.7±0.2	16.9±2.1	nd	nd	nd	nd
2,5-dimethylpyrazine	2.8±0.3	4.4±0.2	4±0.2	nd	8.7±0.4	17.5±0.4	3.2±0.2	10.7±1	nd	nd
2,6-dimethylpyrazine	1.01±0	1.8±0.1	nd	nd	1.1±0.2	5.07±0.5	nd	nd	nd	nd
ethyl-3,6-dimethylpyr[h]	nd	nd	nd	6.3±1.3	3.4±0.2	10.9±1.6	1.7±0.1	4.9±0.5	nd	nd

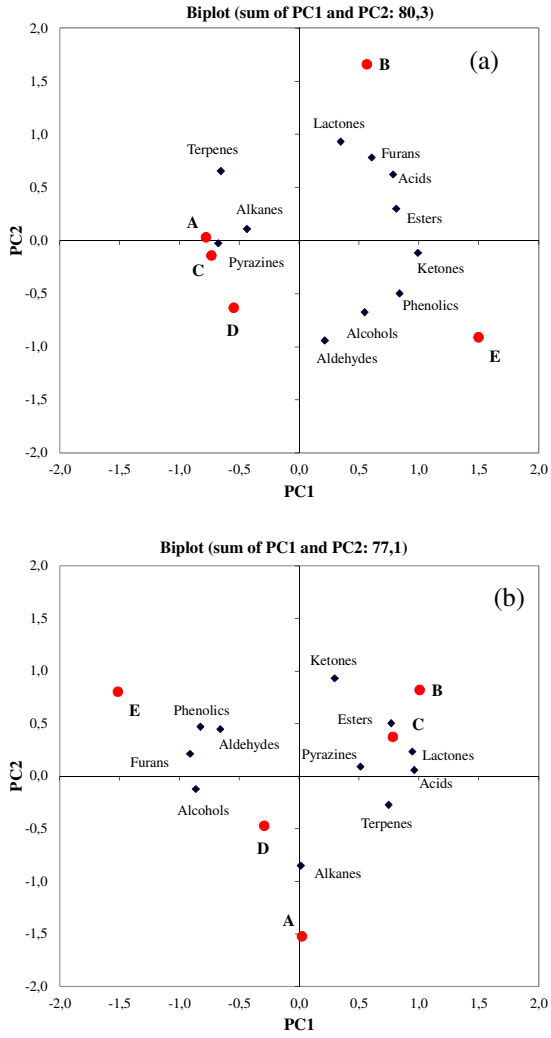
[a]2-methyl propyl ester acetic acid;
[b]1-butanol 3-methyl acetate;
[c]2-methyl ethyl ester propanoic acid;
[d]2-furan carboxilic acid-ethyl ester;
[e]2-methoxy phenol;
[f]3-methyl phenol;
[g]2 methoxy, 4 methyl phenol;
[h]ethyl-3,6-dimethylpyrazine.

nd: not detected.

3.23.4 Principal component analysis

To obtain more detailed information on the VOCs involved in the differentiation of chestnut flour based sourdoughs (Trials A-E), factorial principal component analysis (PCA) was applied to the VOCs classes of sourdoughs. The two first principal components (PCs) were sufficient to explain the maximum variation in all original dates. Figure 27 shows the bidimensional representation (PCA biplot) performed on variables and samples at 24 h (Figure 27a) and 288 h (Figure 27b) of fermentation separately. The results of PCA statistical analysis were projected on two-dimensional plots defined by the first two factors. The first two principal components (PC1 and PC2, Figure 27a) accounted for 80% of the total variance of the data at 24 h. In particular, the PC1 explained 54% of the variation of the VOCs data within the five trials (A-E) and it was clearly defined by major chemical classes of VOCs, while the PC2 explained 26%. At 24 h, sourdoughs could be divided into three clusters: A, C and D grouped together, while B and E were positioned on the positive side of PC1 in separate groups. The first group was associated with alkanes, pyrazines and terpenes. The chestnut flour sourdough (E) proved to be strongly characterized by ketones, phenols, alcohols and aldehydes, while the main VOCs of sourdough B (buckwheat/chestnut flours) were esters, acids, furans and γ-butyrolactone. At 288 h of fermentation (PC1 and PC2, Figure 27b) 77% of the total variance (55% and 22%, respectively) was explained, and a good separation among sourdoughs appeared clearly evident (Figure 26b). B and C were positively scored on PC1, thus being linked to acids, lactones, esters, terpenes, pyrazines and ketones, while E and D appeared in the negative side. With regard to PC2 in the positive side, E was characterized by furans, phenols and aldehydes. In addition, D was characterized by alcohols, while A, in the lower-right quadrant, appeared closely related to alkanes. Therefore, results of PCA revealed a clear influence of fermentation on the volatile composition of sourdoughs.

Figure 27 Results of PCA score and loading biplot for A, B, C, D and E after 24 h (a) and 288 h (b) of fermentation.



3.23.5 Strains technological characterization

All strains were analyzed for biochemical features of technological interest (Table 19). Only strains referable to the species *Pd. lolii* espressed ureolytic activiy. Such results confirmed evidences obtained by Zotta et al. (2007) for lactobacilli isolated from sourdoughs used for the production of the "Cornetto di Matera". According to authors, no strain out of 41 was

able to hydrolyze urea (Zotta et al., 2007). During sourdough fermentation, the production of lactic and acetic acids by LAB promotes a drop in pH values which may affect the metabolic activities of LAB and decrease their performance. The responses of bacteria to acid damage differ. Among the stress response mechanisms, urease activity seems to play a role in protecting some microorganisms from the harmful effect of acid conditions, by increasing the environmental pH as a result of the conversion of urea into ammonia and carbon dioxide (van de Guchte et al., 2002). The relationship of urease activity to pH modulating capacity has been demonstrated in *Helicobacter pylori* (Toledo et al., 2002) and *Streptococcus thermohilus* (Blaiotta et al., 2011).

Table 19 Biochemical characterization of LAB strains isolated from chestnut flour-based mature sourdoughs.

		Activity			EPS production from						Acid from			
Strain	Taxon	Phytasic	Starch hydrolisis	Ureolytic	Glucose	Maltose	Sarch	Saccharose	Fructose	Maltosio	Fructose	Maltose+ Fructose	Arabinose	Xylose
A71	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-
A72	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
A73	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
A74	*Lb. plantarum* group	-	+	-	+	+	-	+	+	+	+	+	-	-
A75	*Lb. plantarum* group	-	+	-	+	+	-	+	+	+	+	+	-	-
A76	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
A77	*Lb. plantarum* group	-	+	-	+	+	-	+	+	+	+	+	-	-
A78	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
A79	*Lb. plantarum* group	-	+	-	+	+	-	+	+	+	+	+	-	-
A710	*Lb. plantarum* group	-	+	-	+	+	-	+	+	+	+	+	-	-
B71	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-
B72	*Pediococcus lolii*	-	-	+	+	+	+	+	+	-	+	+	-	+
B73	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-
B74	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
B75	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-
B76	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-
C71	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
C72	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
C73	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
C74	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
C75	*Lb. plantarum* group	-	+	-	+	+	-	+	+	+	+	+	-	-
C76	*Lb. plantarum* group	-	+	-	+	+	-	+	+	+	+	+	-	-
C77	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
C78	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
C79	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
C710	*Lb. plantarum* group	-	-	-	+	+	-	+	+	+	+	+	-	-
D71	*Pediococcus lolii*	-	-	+	+	+	+	+	+	+	+	+	-	+
D72	*Pediococcus lolii*	-	-	+	+	+	+	+	+	-	+	+	-	+
D73	*Lb. plantarum* group	+	-	-	+	+	-	+	+	+	+	+	-	-
D74	*Lb. plantarum* group	+	-	-	+	+	-	+	+	+	+	+	-	+
D75	*Lb. plantarum* group	+	-	-	+	+	-	+	+	+	+	+	-	-
D76	*Lb. plantarum* group	+	-	-	+	+	-	-	+	+	+	+	-	-
E71	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-
E72	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	+
E73	*Lb. plantarum* group	-	+	-	+	+	-	+	+	+	+	+	+	-
E74	*Lb. plantarum* group	+	-	-	+	+	-	+	+	+	+	+	-	-
E75	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-
F71	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-
F72	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-
F73	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	+
F74	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-
F75	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-
F76	*Lb. plantarum* group	+	-	-	+	+	+	+	+	+	+	+	-	-

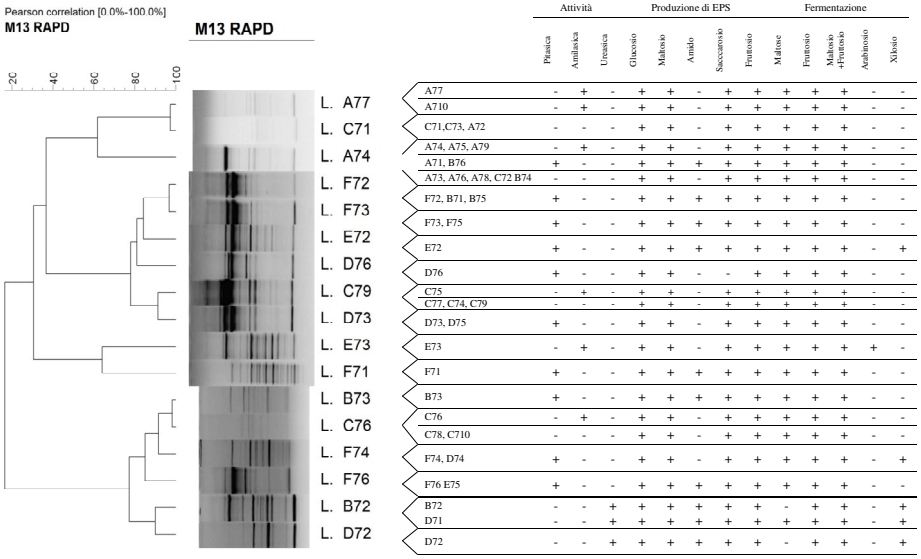
Moreover, none of the tested strains expressed β-glucosidasic activity with precursor used for testing. Actually, the properties of bread may be improved by the hydrolysis of structural and storage polysaccharides. β-glucosidases, a major group of glycosyl hydrolase enzymes, catalyse the selective cleavage of β-1,4-glycosidic linkages of various disaccharides, oligosaccharides, and alkyl-and aryl-β-D-glucosides (Yan et al., 1998). Hydrolysis of glycosidic bonds plays an important role in several biological pathways. *β*-glucosidases are responsible for the hydrolysis of cello-oligosaccharides and cellobiose in the terminal step in the degradation of cellulose, an important fibresource in cereal foods. In addition, these

enzymes hydrolyse toxic and/or bitter glucosides, release aromatic compounds and synthesize various oligosaccharides, glycoconjugates, alkyland amino-glucosides (Bhatia et al., 2002). Results obtained during this survey did not fit the large differences in production of intracellular β-glucosidase found within strains of the *Lb. plantarum* group recorded by Zotta et al. (2007). According to authors, several strains of *Lb. plantarum* exhibited high levels of p-NPG degradation. With reference to phytasic activity, almost 44% of the tested cultures was able to degrade the phytic acid. In cereal grains, most of the total phosphorus is present as phytic acid (myo-inositol hexaphosphate), which is located mainly in the outer layers of the grain. Phytic acid is often regarded as an anti-nutritional compound since it chelates proteins, amino acids and divalent cations such as Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, preventing their absorption by the intestinal mucosa (De Angelis et al., 2003). Because of this, the degradation of phytate by fermentation before the product is baked is desirable. During fermentation phytate-degrading enzymes, which are present in yeasts and LAB isolated from sourdoughs (Lopez et al., 2000), catalyse the stepwise hydrolysis of phytic acid to myo-inositol via penta- to monophosphates or orthophosphates (Reale et al., 2004). In addition, the low pH resulting from acid production by LAB promotes the activation of endogenous flour phytases, and so contributes to further phytate degradation (Lopez et al., 2001; Leenhardt et al., 2005). The 19% of the strains, all belonging to the *Lb. plantarum* group, were able to hydrolyze starch and about the 40% to produce EPS in presence of this compound. On the other hand, all tested strains were able to produce EPS in presence of glucose, maltose, saccharose and fructose. With reference to the use of pentose as the sole carbon source, it must be pointed out that just one strain, *Lb. plantarum* D73, was able to ferment arabinose and six strains, namely D79, B71, B72, B74, E72 and F73, were able to use xylose. All strains were capable to grow in presence of fructose and fructose and maltose, while just two strains, B72 and D73, both belonging to the species *Pd. lolii*, were unable to metabolize maltose.

3.23.6 Comparison between strains' genotypical and biochemical characterization

By strains grouping according to biochemical profile new rearrangements appeared. In order to compare all acquired info, data regarding strain typing at both molecular and phenotypical level were combined (Figure 28). As clearly evident from Figure 28, a sub-stratification could be highlighted. In some cases, i.e. strain E73, the pattern uniqueness is at both levels. In other cases, strains coming from different sourdoughs but characterized by the same DNA fingerprinting even presented the same biochemical profile. This is for instance the case of *Lb. plantarum* strains F74 and D74 or F72, B71 and B75. On the other hand, for strains isolated from wheat containing sourdoughs a higher variability at biochemical level could be recorded. As matter of fact, seven strains with the same RAPD pattern could be discriminated into three different biochemical profiles.

Figure 28 Combined analysis of biochemical and molecular evidences

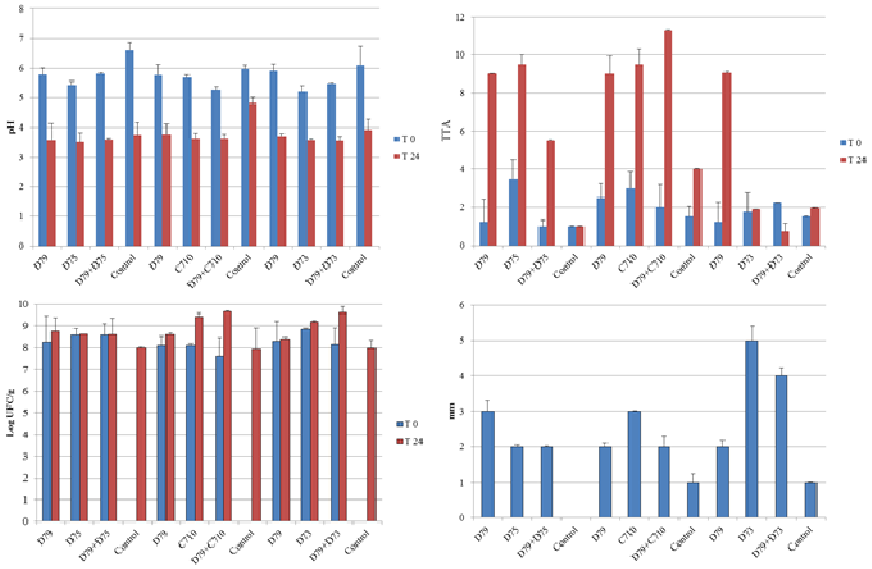


	Attività			Produzione di EPS					Fermentazione				
	Fitasica	Amilaska	Ureasica	Glucosio	Maltosio	Amido	Saccarosio	Fruttosio	Maltose	Fruttosio	Maltosio +Fruttosio	Arabinosio	Xilosio
A77	-	+	-	+	+	-	+	+	+	+	+	-	-
A710	-	+	-	+	+	-	+	+	+	+	+	-	-
C71,C73, A72	-	-	-	+	+	-	+	+	+	+	+	-	-
A74, A75, A79	-	+	-	+	+	-	+	+	+	+	+	-	-
A71, B76	+	-	-	+	+	+	+	+	+	+	+	-	-
A73, A76, A78, C72 B74	-	-	-	+	+	-	+	+	+	+	+	-	-
F72, B71, B75	+	-	-	+	+	+	+	+	+	+	+	-	-
F73, F75	+	-	-	+	+	+	+	+	+	+	+	-	-
E72	+	-	-	+	+	+	+	+	+	+	+	-	+
D76	+	-	-	+	+	-	-	+	+	+	+	-	-
C75	-	+	-	+	+	-	+	+	+	+	+	-	-
C77, C74, C79	-	-	-	+	+	-	+	+	+	+	+	-	-
D73, D75	+	-	-	+	+	-	+	+	+	+	+	-	-
E73	-	+	-	+	+	-	+	+	+	+	+	+	-
F71	+	-	-	+	+	+	+	+	+	+	+	-	-
B73	+	-	-	+	+	+	+	+	+	+	+	-	-
C76	-	+	-	+	+	-	+	+	+	+	+	-	-
C78, C710	-	-	-	+	+	-	+	+	+	+	+	-	-
F74, D74	+	-	-	+	+	-	+	+	+	+	+	-	+
F76 E75	+	-	-	+	+	+	+	+	+	+	+	-	-
B72	-	-	+	+	+	+	+	+	-	+	+	-	+
D71	-	-	+	+	+	+	+	+	+	+	+	-	+
D72	-	-	+	+	+	+	+	+	-	+	+	-	+

3.23.7 Evaluation of the fermentative performance of three multiple starter

Three LAB strains were selected on the basis of evidences retrieved by combining biochemical features with molecular strain typing. In detail, *Pd. Lolii* B72, a strain unable to ferment maltose was combined with *Lb. plantarum*E75, with phytsase activity, or *Lb. plantarum* C710 unable to produce EPS by starch, or with *Lb. plantarum* E72, a strain capable to ferment xylose. Flours blend (chestnut flour/rice flour, 40/60) was mixed with a cell suspension and incubated at 30°C for 24 h. pH, TTA, LAB loads and volumes increasing were evaluated at time zero and after 24 h (Figure 29).

Figure 29 Results concerning pH, TTA, LAB loads and volumes increasing (time zero and after 24 h at 30°C) of the three selected combinations of LAB strains.



Initial pH values were around 6.00 at the beginning of the trial and dropped at about 4.00 in all cases. Values recorded when pediococci and lactobacilli were combined, resulted always lower than pH values reached by lactobacilli as single strains, thus confirming the contribute of homo-fermentative strains. In all, cases, in 24 h, pH dropped to values around 4.00, that are to be considered critical for microbial activity (Gobbetti et al.,2005). These results may be put in relation with the temperature used for trials (30°C), that is enough high to favor LAB instead of yeasts. Analogous considerations for TTA, even if, the expected highest TTA values in co-presence of pediococci and lactobacilli could be retrieved just for combination between strains B72 and C710. In the mix E73-B72, the low value of TTA reached by strain E73 affected results concerning the blend with *Pd. lolii* B72, too. On the other hand, in this combination the best results in terms of leavening were recorded. In all trials, microbial loads increased of about one Log CFU g^{-1}. As expected, since flours were not sterilized, LAB population level in controls, that was at time zero under the detection limit of the counting method, arrived up to about 8 Log CFU g^{-1} in 24 h. Such phenomenon may be related to the possibility that these populations are present in viable non culturable (VNC) state, due to the carbohydrate starvation (Iacumin et al., 2009).

3.24 Conclusions

This work describes for the first time the development of a stable biota in spontaneously fermented chestnut-flour-based sourdoughs (including GF), propagated under technological conditions similar to those used for the production of industrial wheat and rye sourdoughs. All flours used in this study are commercially available. Hence, the research findings can be adapted to industrial bread production. The dominant retrieved species basically overlap with those commonly associated with traditional wheat sourdoughs; even if species, which are more commonly isolated from tropical fermentation products, such as *Pd. Pentosaceus* or *W. cibaria*, were identified as well. In spite of the poor speciography, according to M13 RAPD, a high genotypic biodiversity could be retrieved in all combinations, except the one with wheat flour, confirming that GF sourdoughs may be considered an important reservoir of novel and competitive LAB strains. With regard to the aroma characterization, 59 volatile compounds were identified in sourdoughs obtained from 100% chestnut flour and chestnut flour blends during fermentation. The VOCs profile of all sourdoughs were dominated by alcohols, esters, acids, aldehydes and ketones, all well-known flavour compounds in sourdough fermentation. PCA offered a good separation of the samples and pointed out the effect of microbial fermentation on sourdough VOCs. Such wide variety of volatile metabolites may obviously contribute to enrich flavour, taste and aroma of bread or other leavened bakery products. Moreover, the identification of the principal compounds produced during the fermentation process can be of help in the search of fermentation indices. In general terms, the present work may support technologists in the development and improvement of highly nutritional GF chestnut-based bakery products, by providing basic knowledge on the impact of flours obtained from different botanical sources. Further studies are currently in progress to evaluate the influence of the isolated LAB strains on the technological and sensorial quality of GF bread.

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Appendix 1 List of publications

Blaiotta G, Di Capua M, Coppola R, Aponte M (2012) Production of fermented chestnut purees by lactic acid bacteria. Int J Food Microbiol 158:195-202.doi: 10.1016/j.ijfoodmicro.2012.07.012.

Blaiotta G, Di Capua M, Romano A, Coppola R, Aponte M (2013) Optimization of water curing for the preservation of chestnuts (Castanea Sativa mill.) and evaluation of microbial dynamics during process by means of a polyphasic approach. Food Microbiol 42:47-55. doi: 10.1016/j.fm.2014.02.009.

Blaiotta G, La Gatta B, Di Capua M, Di Luccia A, Coppola R, Aponte M (2013) Effect of chestnut extract and chestnut fiber on viability of potential probiotic Lactobacillus strains under gastrointestinal tract conditions. Food Microbiol 36:161-169.doi: 10.1016/j.fm.2013.05.002

Appendix 2 List of abstract

Di Capua M. (2011) Sustainable Technological Innovation of the Cestnut Processing Chain. 16thWorkshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology (Tecniche nuove, eds.) pp. 323-324, Univ. of Milano and Piacenza, Lodi.

Di Capua M(2012) Effect of Chestnut Extract obtained by "Castagna di Montella" on the viability of potential probiotic strains and application to the production of a functional chestnut Mousse. 17thWorkshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology (Il Ponte Vecchio ed.) pp. 215-216, Univ. of Bologna, Cesena. ISBN 9788865412244

Di Capua M, Blaiotta G, Di Luccia A, Coppola R, Aponte M (2012) Effect of chestnut extract and chestnut fiber on viability of potential prebiotic strains uder gastrointestractinal simulated conditions. III Convegno Nazionale "Società Italiana di Microbiologia Agraria, Alimentare e Ambientale" Bari.